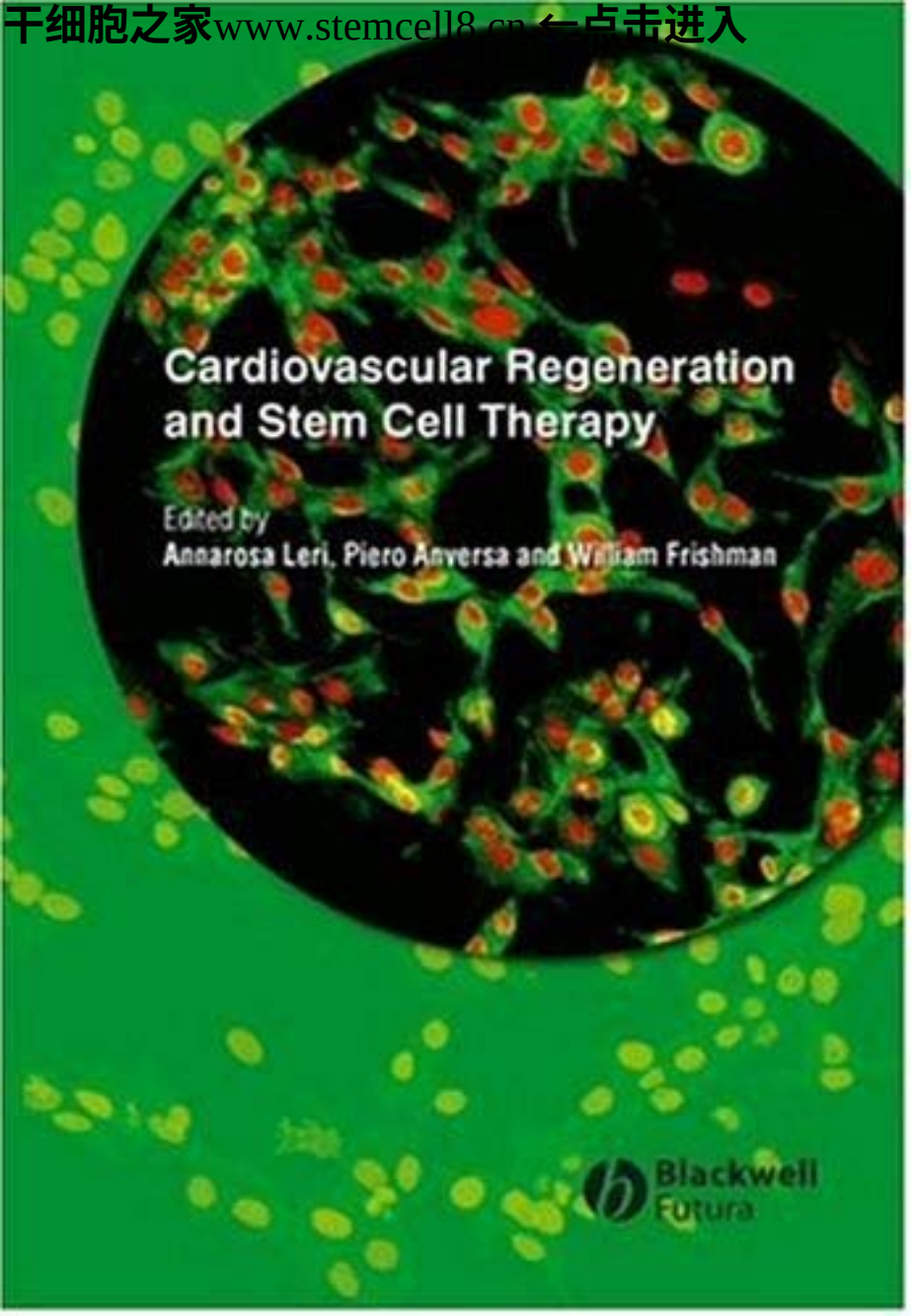


Cardiovascular Regeneration and Stem Cell Therapy

Dedication

To all those investigators who seek the truth in biomedical science to improve patient care.

Cardiovascular Regeneration and Stem Cell Therapy

EDITED BY

Annarosa Leri, MD

Professor of Medicine
Department of Medicine
Cardiovascular Research Institute
New York Medical College
Valhalla, NY

Piero Anversa, MD

Professor of Medicine and Vice Chairman
Department of Medicine
Director, Cardiovascular Research Institute
New York Medical College
Valhalla, NY

William H. Frishman, MD

Rosenthal Professor and Chairman
Department of Medicine
New York Medical College
Director of Medicine, Westchester Medical Center
Valhalla, NY

Blackwell Futura

Blackwell Futura is an imprint of Blackwell Publishing

Blackwell Publishing, Inc., 350 Main Street, Malden, Massachusetts 02148-5020, USA
Blackwell Publishing Ltd, 9600 Garsington Road, Oxford OX4 2DQ, UK
Blackwell Science Asia Pty Ltd, 550 Swanston Street, Carlton, Victoria 3053, Australia

First published 2007

1 2007

ISBN: 978-1-4051-4842-9

Library of Congress Cataloging-in-Publication Data

Cardiovascular regeneration and stem cell therapy / edited by
Annarosa Leri, Piero Anversa, William Frishman.
p. ; cm.
Includes index.
ISBN-13: 978-1-4051-4842-9 (alk. paper) 1. Heart–Regeneration.
2. Stem cells–Transplantation. I. Leri, Annarosa. II. Anversa, Piero.
III. Frishman, William H., 1946–
[DNLM: 1. Cardiovascular Diseases–therapy. 2. Regenerative
Medicine. 3. Stem Cell Transplantation. WG 166 C26669 2007]

RC669.C2882 2007
616.1′06–dc22

2006030356

A catalogue record for this title is available from the British Library

Acquisitions: Gina Almond
Development: Fiona Pattison
Set in 9.5/12pt Minion and Frutiger by TechBooks, New Delhi, India
Printed and bound in Singapore by Markono Print Media Pte Ltd.

For further information on Blackwell Publishing, visit our website:
www.blackwellfutura.com

The publisher's policy is to use permanent paper from mills that operate a sustainable forestry policy, and which has been manufactured from pulp processed using acid-free and elementary chlorine-free practices. Furthermore, the publisher ensures that the text paper and cover board used have met acceptable environmental accreditation standards.

Contents

Contributors

Mehrdad Abedi, MD
Roger Williams Medical Center
Department of Research
Providence, RI

Piero Anversa, MD
Professor & Vice Chairman
Department of Medicine
Vice Chairman Department of Medicine
New York Medical College
Director, Cardiovascular Research Institute
Valhalla, NY

Takayuki Asahara, MD, PhD
Department of Regenerative Medicine Science,
Tokai University School of Medicine; *and*
Department of Regenerative Medicine
Institute of Biomedical Research and Innovation; *and*
Laboratory for Stem Cell Translational Research
RIKEN Center for Developmental Biology
Kobe, Japan

Claudia Bearzi
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Remus Berretta, BS
Department of Physiology
Cardiovascular Research Center
Temple University School of Medicine
Philadelphia, PA

Roberto Bolli, MD
Chief, Division of Cardiology
Institute of Molecular Cardiology
University of Louisville, School of Medicine
Louisville, KY

Alessandro Boni PhD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Maurizio C. Capogrossi, MD
laboratorio di Patologia Vascolare
Istituto Dermopatico dell'Immacolata-IRCCS
Rome, Italy

Xiongwen Chen, PhD
Department of Physiology
Cardiovascular Research Center
Temple University School of Medicine
Philadelphia, PA

Gerald Colvin, DO
Rhode Insland Hospital
Department of Hematology/Oncology
Providence, RI

Ayelet Dar, PhD
Department of Immunology
Weizmann Institute
Rehovot, Israel

Antonella De Angelis PhD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Stefanie Dimmeler, PhD
Medizinische Klinik III (Kardiologie)
University of Frankfurt (Molecular Cardiology)
Johann-Wolfgang Goethe-Universität
Frankfurt am Main
Germany

William H. Frishman, MD
Professor and Chairman
Department of Medicine
New York Medical College
Director of Medicine
Westchester Medical Center
Valhalla, NY

Jun Fujita, MD, PhD
Department of Regenerative Medicine and
Advanced Cardiac Therapeutics
Keio University School of Medicine
Tokyo, Japan

Keiichi Fukuda, MD, PhD, FACC
Professor and Chair
Department of Regenerative Medicine and
Advanced Cardiac Therapeutics
Keio University School of Medicine
Tokyo, Japan

Antonia Germani, PhD
Laboratorio di Biologia Vascolare e Terapia Genica
Centro Cardiologico Monzino, IRCCS
Milan, Italy

Marco Giorgio PhD
Department of Experimental Biology
European Institute of Oncology
Milan, Italy

Daihiko Hakuno, MD, PhD
Department of Regenerative Medicine and
Advanced Cardiac Therapeutics
Keio University School of Medicine
Tokyo, Japan

Toru Hosoda PhD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Steven R. Houser, PhD
Professor, Department of Physiology
Temple University School of Medicine
Philadelphia, PA

Marjan Jahangiri, MS, FRCS (CTh)
Department of Cardiothoracic Surgery and
Cardiac and Vascular Sciences
St George's Hospital and Medical School
London, UK

Jan Kajstura PhD
Professor of Medicine
Cardiovascular Research Institute
New York Medical College
Valhalla, NY

Timothy J. Kamp, MD, PhD
Associate Professor of Medicine and Physiology;
Codirector Regenerative Medicine Program
Division of Cardiovascular Medicine
University of Wisconsin-Madison; *and*
WiCell Research Institute
Madison, WI

Raj Kishore, PhD
Assistant Professor of Medicine
Tufts University School of Medicine
St. Elizabeths Medical Centre
Boston, MA

Orit Kollet, PhD
Department of Immunology
Weizmann Institute
Rehovot, Israel

Issei Komuro, MD, PhD
Department of Cardiovascular Science and Medicine
Chiba University Graduate School of Medicine
Chiba, Japan

Masamichi Koyanagi, MD
Medizinische Klinik III (Kardiologie)
University of Franfurt (Molecular Cardiology)
Johann-Wolfgang Goethe-Universität
Frankfurt am Main, Germany

Hajime Kubo, PhD
Department of Physiology
Cardiovascular Research Center
Temple University School of Medicine
Philadelphia, PA

Tsvee Lapidot, PhD
Department of Immunology
Weizmann Institute
Rehovot, Israel

Nicole LeCapitaine PhD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Richard T. Lee, MD
Associate Professor
Brigham and Women's Hospital
Cambridge, MA

Annarosa Leri, MD
Professor of Medicine
Department of Medicine
New York Medical College
Cardiovascular Research Institute
Valhalla, NY

Ronglih Liao, PhD
Associate Professor
Cardiac Muscle Research Laboratory
Cardiovascular Division
Department of Medicine
Brigham and Women's Hospital
Harvard Medical School
Boston, MA

Federica Limana, PhD
Laboratorio di Biologia Vascolare e Terapia Genica
Centro Cardiologico Monzino-IRCCS
Milan, Italy

Maria Loredo MD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Douglas W. Losordo, MD
Director, Feinberg Cardiouascular
Research Institute
Professor of Medicine
Northwestern University
Chicago, Il.

Gary E. Lyons, PhD
Department of Anatomy
University of Wisconsin-Madison
Madison, WI

Shinji Makino, MD, PhD
Department of Regenerative Medicine and
Advanced Cardiac Therapeutics
Keio University School of Medicine
Shinjuku-ku
Japan

Kaushik Mandal, MD
Department of Cardiothoracic Surgery
St George's Hospital and Medical School
London, UK

Thomas S. Mitchell MD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Frederic Mouquet, MD, PhD
Cardiac Muscle Research Laboratory
Cardiovascular Division
Department of Medicine
Brigham and Women's Hospital
Harvard Medical School
Boston, MA

Angelo Nascimbene MD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Pier Giuseppe Pelicci PhD
Department of Experimental Biology
European Institute of Oncology
Milan, Italy

Marc A. Pfeffer, MD, PhD
Professor of Medicine
Department of Medicine, Division of Cardiology
Brigham and Women's Hospital
Boston, MA

Otmar Pfister, MD
Department of Research, Division of Cardiology
University Hospital Basel
Switzerland

Peter J. Quesenberry, MD
Director, Division of Hematology
and Oncology
Rhode Island Hospital
Providence, RI

Robert C. Robbins, MD
Professor and Chair of Cardiothoracic Surgery
Stanford University Medical Center
Stanford, CA

Nadia Rosenthal, PhD
Head and Coordinator, Mouse Biology Unit
EMBL-Monterotondo Outstation, Monterotondo
Rome, Italy

Marcello Rota PhD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Francesco Rotatori MD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Maria Paola Santini PhD
Hospital Bambino Gesu'
Autoimmunity and Tissue Regeneration Laboratory
Rome, Italy

Vincent F.M. Segers, MD
Department of Medicine
Brigham and Women's Hospital
Harvard Medical School
Cambridge, MA

Ahmad Y. Sheikh, MD
Department of Cardiothoracic Surgery
Stanford University Medical Center
Stanford, CA

Robert Siggins PhD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Edmund H. Sonnenblick, MD
Safra Distinguished Professor of Medicine,
Albest Einstein College of Medicine, Bronx, NY
Visiting Professor of Medicine
New York Medical College
Valhalla, NY

Mark A. Sussman, PhD
San Diego State University Heart Institute; *and*
Department of Biology
San Diego, CA

Hiroyuki Takano, MD, PhD
Department of Cardiovascular Science and Medicine
Chiba University Graduate School of Medicine
Chiba, Japan

Konrad Urbanek MD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Carmen Urbich PhD
Medizinische Klinik III (Kardiologie)
University of Frankfurt (Molecular Cardiology)
Johann-Woelgang Goethe-Universität
Frankfurt am Main, Germany

Susanna Valentini MD
Cardiovascular Research Institute
Department of Medicine
New York Medical College
Valhalla, NY

Rachel Wilson
Department of Physiology
Cardiovascular Research Center
Temple University School of Medicine
Philadelphia, PA

Nadine Winn PhD
Mouse Biology Unit
EMBL-Monterotondo Outstation, Monterotondo
Rome, Italy

Joseph C. Wu, MD, PhD
Department of Medicine, Division of Cardiology; *and*
Department of Radiology, Division of Nuclear Medicine
Stanford University Medical Center
Stanford, CA

Qingbo Xu, MD, PhD
BHF John Parker Chair of Cardiovascular Sciences
Cardiovascular Division
King's College London
London, UK

Phillip C. Yang, MD
Department of Medicine, Division of Cardiology
Stanford University Medical Center
Stanford, CA

Andreas M. Zeiher, MD
Director, Medizinische Klinik III (Kardiologie)
University of Frankfurt (Molecular Cardiology)
Johann-Wolfgang Goethe-Universität
Frankfurt am Main
Germany

Preface

The purpose of this book is to bring together groups of scholarly articles articles from international experts that discuss both the current state of knowledge regarding regenerative cardiology and the possibilities for using cell replacement therapy, with and without adjuncts, as a clinical approach for treating cardiovascular diseases, specifically myocardial infarction and cardiomyopathy. Cell replacement therapy may also have a future role in benefiting the course of cardiovascular aging.

The book is organized into five sections. The first section provides detailed discussions of stem cell biology, including the properties of homing and transdifferentiation, and focuses on the types of cells being studied for use in myocardial and vascular regeneration.

The second section is organized to provide a thorough overview of cardiac progenitor cells in the adult heart, focusing in on their biologic characteristics and function. Their recent discovery and characterization helps to confirm that the heart is not a postmitotic organ as previously thought, but an organ capable of regeneration and self-repair. Heart homeostasis is regulated by a stem cell compartment characterized by multipotent cardiac cells that possess the ability to acquire the distinct cell lineages of the myocardium.

The third section provides the experimental and clinical evidence that stem cells play a role in myocardial and vascular regeneration. A discussion is also included of various biologic factors (cytokines, IGF-1) that can both amplify and modify the regenerative processes in the heart.

The fourth section critically reviews experimental studies in heart failure that have used stem cell therapy in diabetic cardiomyopathy, myocardial aging, and extensive ischemic injury. Some of the strategies being utilized to enhance cell replacement therapy are also highlighted, including the use of bioengineered scaffolds and the application of gene therapy.

The final section consists of a chapter discussing the future directions of regenerative cardiovascular medicine in disease treatment. It was recently demonstrated that a human urinary bladder can be regenerated outside the body from stem cells, grown on biological scaffolds, and implanted as a functioning organ in humans. This approach may not be far away in human beings with cardiovascular diseases. Currently, stem cells are being given in clinical trials as a peripheral or direct coronary artery infusion or are being implanted directly into the heart with early evidence of clinical benefit. Various growth factors are also being used as adjuncts to stimulate the mobilization, homing, and growth of peripheral and/or organ-specific stem cells for myocardial repair.

The future is bright for utilizing and amplifying the biologic processes of myocardial and vascular regeneration for the treatment of human disease. This book provides a window into that future.

The editors are indebted to the many contributors to this book who come from various scientific and clinical disciplines and to our trainees and colleagues who have served as research collaborators and sources of intellectual stimulation. A special acknowledgment must be given to Joanne Cioffi-Pryor who has served as the in-house editorial assistant for this book and for other cardiovascular texts and journals from our institution. Her critical eye and meticulous attention to detail have contributed to the successful completion of this text.

We wish to acknowledge our editor, Gina Almond, and the editorial staff at Blackwell, especially Fiona Pattison, who have guided the book through the editorial process.

Finally, we are privileged to bring together this unique and timely book on this new and burgeoning field of scientific and clinical endeavor, regenerative cardiovascular medicine. The editors and contributors hope that this text will increase the understanding of cardiovascular regeneration so as to stimulate an ongoing dialogue between scientists, clinicians, and other interested individuals as this field continues to move forward.

Annarosa Leri, MD
Piero Anversa, MD
William H. Frishman, MD
Valhalla, NY

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

Introduction

Annarosa Leri, MD*, Piero Anversa,* MD*, & William H. Frishman,* MD

Cardiovascular diseases, including myocardial infarction and heart failure, are the leading causes of death in the industrialized world and account for 16.7 million deaths annually. In the United States, approximately 900,000 people die every year from the complications of cardiovascular diseases, representing 38% of the overall mortality in this country (www.americanheart.org/statistics). The epidemic problem of heart failure, together with the limitations in its management, constitute the basis for the current interest in regenerative cardiology. This novel field aims at the identification of primitive cells and/or gene products, which are capable of activating directly or indirectly the formation of myocytes and coronary vessels lost because of pathological conditions. Within a few years following the experimental documentation that bone-marrow-derived cells (BMDCs) could induce cardiac repair after infarction, clinicians began to administer endothelial progenitor cells, mononuclear bone marrow cells (BMCs), and CD34-positive cells to patients affected by acute and chronic ischemic heart failure [1–5]. In general, these interventions have had a favorable outcome, pointing to the feasibility and safety of this therapeutic approach. Questions persist, however, concerning the transient and long-term efficacy of this strategy [6–9]. While patients are currently being enrolled in large randomized clinical trials, the documentation for the existence of cardiac-specific adult progenitor cells (CPCs) has also raised the possibility of using these resident undifferentiated cells for the treatment of human disease [10, 11].

The notion that the reconstitution of damaged infarcted myocardium cannot be accomplished has been successfully challenged by experimental studies in animals, by using the intramyocardial injection of c-kit-+ BMDCs, mesenchymal stem cells, embryonic stem cells, and clonogenic c-kit-+ CPCs, by using the systemic mobilization of BMDCs, and the local activation and mobilization of CPCs with growth factors [12]. In all cases, a significant regeneration of infarcted myocardium has been obtained in combination with an improvement in cardiac function and a decreased animal mortality. In addition, areas of spontaneous myocardial regeneration, mediated by activation and differentiation of resident CPCs, have been found in the infarcted human heart [12].

Clinically, it might be more efficient and powerful to employ stem cells that reside in the damaged organ than to deliver cells from the bone marrow and other sources. By necessity, noncardiac progenitor cells have to undergo a reprogramming phase to acquire the cardiomyocyte lineage and to generate coronary vessels [13]. However, exogenous cells can be easily collected from a bone marrow biopsy or the peripheral blood, offering a unique and simple supply of potentially efficacious cells for use in cardiac repair. At present, it is unknown whether CPCs and BMCs are similarly effective in reconstituting dead myocardium. Limitations exist in CPC growth in one case and in BMDC transdifferentiation in the other. The natural pathways of differentiation and stability of the original phenotype are preserved when stem cells are located in their developmentally determined microenvironment [14]. However, the inter- and intragerm layer transitions of stem cells also depend on changes in the local milieu that condition their ability to differentiate into end-stage effector cells, typical of the injured organ [13, 14]. CPCs are predestined to become myocardial cells, and this inherent advantage overcomes the need for the complex and time-consuming process of chromatin reorganization. Whether these differences have an

impact on the timing and efficiency of myocardial regeneration by various cell types is presently unknown. The rapid restoration of myocardium and myocardial functioning is often crucial for the survival of the organ and organism, especially in patients with large myocardial infarcts. Thus, the search continues to find the most appropriate cell type to use in optimizing replacement therapy for the recovery of myocardial structure and function.

References

1 Losordo D, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease. Part II: cell-based therapies. Circulation 2004; 109:2692–2697.

2 Wollert KC, Drexler H. Clinical applications of stem cells for the heart. Circ Res 2005; 96:151–163.

3 Schächinger V, Torsten T, Dimmeler S, Zeiher AM. Bone-marrow-derived progenitor cell therapy in need of proof of concept: design of the REPAIR-AMI trial. Nat Clin Pract Cardiovasc Med 2006; 3(suppl 1):S23–S28.

4 Assmus B, Honold J, Schachinger V, et al. Transcoronary transplantation of progenitor cells after myocardial infarction. *N Engl J Med* 2006; 355(12): 1222-1232,

5 Schachinger V, Erbs S, Elsasser A, et al for the REPAIR-AMI Investigators: Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 2006; 355(12):1210–1221,

6 Britten MB, Assmus B, Abolmaali ND, et al. Preserved functional improvement and evidence for reverse left ventricular remodeling two years after intracoronary progenitor cell therapy in patients with acute myocardial infarction. Circulation 2005; 112:ii–632.

7 Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (bone marrow transfer to enhance ST-elevation infarct regeneration) trial. Circulation 2006; 113:1287–1294.

8 Welt FG, Losordo D. Cell therapy for acute myocardial infarction: curb your enthusiasm? Circulation 2006; 113:1272–1274.

9 Boyle AJ, Schulman SP, Hare JM: Stem cell therapy for cardiac repair. Ready for the next step. *Circulation* 2006; 114:339–352.

10 Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 2006; 113:1451–1463.

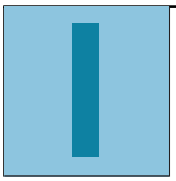
11 Anversa P, Leri A, Kajstura J: Cardiac regeneration. *J Am Coll Cardiol* 2006; 47:1769–1776.

12 Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005; 85:1373–1416.

13 Pomerantz J, Blau HM. Nuclear reprogramming: a key to stem cell function in regenerative medicine. Nat Cell Biol 2004; 6:810–816.

14 Leri A, Kajstura J, Anversa P. Identity deception: not a crime for a stem cell. Physiology 2005; 20:162–168.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman



PART I

Stem cell biology

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

1

CHAPTER 1

Homing of stem cells and tissue injury

Ayelet Dar, PhD, *Orit Kollet,* PhD, *& Tsvee Lapidot,* PhD

Introduction

Hematopoietic stem cells continuously replenish the blood with immature and maturing leukocytes as part of homeostasis. Organ injury dramatically amplifies this process by the secretion of stress signals, which induce recruitment of progenitor and maturing cells from the bone marrow reservoir of leukocytes to the damaged tissue, as part of host defense and repair mechanisms. This chapter will review the reciprocal cross talk between injured tissues and the bone marrow reservoir and will point out key players in stem cell homing. The central roles of the chemokine SDF-1 (CXCL12) and its receptor CXCR4 in stem cell recruitment to inflamed/damaged tissues will be discussed.

The homing process: the roles of SDF-1 and CXCR4

Organ injury and/or inflammation because of viral or bacterial infections are accompanied by an increase in the levels of inflammatory cytokines and chemokines in the damaged organs and consequently in the peripheral blood [1]. These stress signals have been shown to recruit immature hematopoietic stem and progenitor cells as well as maturing leukocytes from the bone marrow reservoir to the circulation, which then home to the damaged liver, brain, heart and other nonhematopoietic organs [2–4], as part of host defense and repair mechanisms. The bone marrow contains a variety of cell types, including hematopoietic stem cells with self-renewal and multilineage differentiation capacity [5, 6] and nonhematopoietic stemcells, mesenchymal stem cells [7] and endothelial progenitor cells [8]. These bone marrow cells have been shown to contribute to tissue regeneration and to the recovery of damaged organs [3, 9] as well as to tissue neovascularization [8, 10].

Several physiological (e.g., physical activity [11]) and pathological (e.g., myocardial infarction, ischemia [12]) stimuli as well as clinical treatments (e.g., granulocyte-colony-stimulating factor [13], statins [14], estrogens [15]) increase the numbers of various bone marrow progenitor cell types in the circulation, with the potential of their migration to injured tissues. Major players in the regulation of this multistep process of cell mobilization and homing are the chemokine SDF-1 and its receptor CXCR4. SDF-1 (also termed CXCL12) is the only known powerful chemoattractant of murine [5] and human [16] hematopoietic stem cells. In early developmental stages, experimental deficiency of SDF-1 results in lethal cardiac defects, similar to those of CXCR4-deficient mice. CXCR4 and its ligand SDF-1 are constitutively expressed by murine and human bone marrow endothelial and endosteal bone lining stromal cells [6, 17–19], which both define the hematopoietic stem cell niches [20, 21]. Homeostatic expression of SDF-1 is also found in nonhematopoietic tissues, including skin [22], epithelial cells in the bile ducts of brain endothelium [2], liver [3], and heart [23, 24].

Many studies document the central roles of CXCR4 in navigating the homing of circulating human $CD34^+$ hematopoietic stem and progenitor cells through the blood-marrow barrier into their specialized niches in the bone marrow. This specific process is induced in response to presentation

of murine SDF-1 (which is cross-reactive with the human chemokine) on endothelial and other bone lining stromal cells in transplanted immune-deficient NOD/SCID mice (reviewed in [25]).

Certain stress-induced physiological and pathological conditions are characterized by SDF-1 elevation in the peripheral blood and within damaged organs, which contribute to the recruitment of $CXCR4^+$ homing cells. For example, after myocardial infarction, SDF-1 mRNA levels are markedly up-regulated in the murine heart and are involved in the chemoattraction of bone-marrow-derived cells [24]. In another model of ischemic cardiomyopathy in the rat heart, it was shown that forced expression of SDF-1 improves ventricular function post damage [26]. In addition, during focal cerebral ischemia, SDF-1 expression is increased in endothelial cells located within the lesioned brain areas and is assumed to induce CXCR4-dependent infiltration of circulating leukocytes [2]. A hint for the contribution of bone-marrow-derived $CD34^+$ progenitor cells to organ recovery was demonstrated using a brain stroke model in mice. The administration of human cord-blood-derived $CD34^+$ cells to mice that were previously subjected to stroke induced neovascularization by vascular endothelial growth factor (VEGF) secretion in the ischemic zone, provided a favorable environment for neuronal progenitor migration and regeneration [4]. In agreement with these observations, it was shown that during hypoxic conditions, the elevation of the transcription factor hypoxia-inducible-factor-1 in endothelial cells selectively up-regulates SDF-1 expression within ischemic regions *in vivo*, which in turn increases the recruitment of $CXCR4^+$ circulating progenitors into areas of reduced oxygen tension in murine skin, muscle, and bone marrow [10]. Furthermore, stress-induced signals such as inflammation, irradiation and hepatitis C virus infection of the murine or human liver result in elevation of SDF-1 amounts, accompanied by HGF (hepatocyte growth factor) expression, which then target human $CD34^+$ progenitors to the damaged liver [3]. Altogether, these results suggest a mechanism for tissue defense. There is also evidence for repair and regeneration by SDF-1-mediated recruitment of $CXCR4^+$ hematopoietic and endothelial precursors upon stress-induced conditions. Interestingly, selective expression of different SDF-1 isoforms has been reported. For example, in the brain, neurons express SDF-1-α, while endothelial cells selectively express SDF-1-β. During cerebral ischemia, transient and selective modulations in SDF-1 expression are believed to regulate distinct pathways for neuronal phenotype or cerebral infiltration [2]. In addition, although α and γ isoforms (but not SDF-1-β) of SDF-1 are abundantly expressed in heart tissue SDF-1, the amounts of SDF-1-α are selectively up-regulated after myocardial infarction [27]. Such modulations in SDF-1 isoform expression imply a specific functional role for different SDF-1 alternative splicing products, which has to be elucidated more broadly.

Inflammation and ischemic and hypoxic conditions are known to stimulate elevation in VEGF and SDF-1 levels [12, 28]. Both SDF-1 and VEGF are reported to be involved in sprouting and remodeling of preexisting blood vessels in the course of angiogenesis and mediate neovascularization [29, 30], for example, by recruiting endothelial cell precursors from the bone marrow [31], for wound healing or in pathological processes such as chronic inflammation or tumor growth [32].

Clinical protocols of DNA-damaging agents such as total body irradiation or chemotherapy have been shown to cause significant increase in SDF-1 levels in the bone marrow and spleen within 24–48 hours, leading to improved CXCR4-dependent homing of human $CD34^+$ stem and progenitor cells in transplanted NOD/SCID mice [17]. In contrast, repetitive administration of G-CSF (granuloycyte colony-stimulating factor), which is widely used in clinical protocols aimed at hematopoietic stem cell mobilization, markedly decreases SDF-1 expression in human and murine bone marrow [13]. Moreover, administration of the sulfated polysaccharide fucoidan, which competes with SDF-1 binding to heparan sulfate, resulted in a rapid and massive release of SDF-1 into the circulation, reduction in its levels in the bone marrow, and a significant increase in the levels of circulating hematopoietic stem and progenitor cells [33]. Supporting this notion, enforced increased levels of SDF-1 by adenoviral vectors [31], MetSDF-1 [34], or SDF-1 injections [35] lead to progenitor and stem cell mobilization. In another model of parabiotic mice with joint circulation, the dramatic elevation in the levels of G-CSF-mobilized stem cells correlated with increased repopulation

in the partner bone marrow, revealing that mobilized stem cells can efficiently home back to G-CSF-simulated bone marrow (mimicking stress injury and inflammation), in contrast to the low homing levels observed with steady state circulating stem cells to nonstimulated bone marrow [36]. This report demonstrates that during stress-induced mobilization, homing rates to the stressed organs are also augmented, confirming that mobilization and homing are sequential, physiological processes. Collectively, these results suggest that the up-regulation of SDF-1 in the injured organ and consequently in the circulation is a prerequisite first step, interfering with the steady state balance in the bone marrow reservoir, initiating mobilization and recruitment, which requires microenvironmental changes of the stem cell niche and its residents—the stem cells.

How do the distant injured organs transfer SDF-1 "stress signals" through the circulation into the bone marrow? The endothelium harbors a highly selective transport system for delivery of chemokines and other molecules across this mechanical barrier—a mechanism termed transcytosis—in which active transfer of proteins and molecules is mediated by transport vesicles (e.g., clathrin-coated pits, caveolae [37]). As such, endothelial cells in the blood-marrow barrier use their CXCR4 receptors to actively regulate SDF-1 levels by CXCR4-mediated functional transcytosis of this chemokine from the circulation into the bone marrow. This unique and potent capacity also characterizes other stromal cells, including cells of the endosteum region, which comprise the hematopoietic stem cell niche, but not bone marrow residing or circulating hematopoietic cells [19]. It is noteworthy that CXCR4-mediated translocation of functional circulating SDF-1 into the bone marrow can actively increase hematopoietic progenitor cell homing to this tissue, which is followed by stem cell mobilization and recruitment to injured organs. Importantly, this process uniquely defines the dual role of CXCR4-expressing tissue-anchored stromal cells in the bone marrow, spleen, and other organs. CXCR4 is capable of activating signaling pathways upon SDF-1 stimulation (e.g., migration, proliferation, proteolytic enzyme secretion, angiogenesis, and neovascularization) and can also regulate internalization and trafficking of its ligand potentially aimed at communication between organs and the bone marrow reservoir [19, 38].

Homing and the injured myocardium

SDF-1 appears to be a key factor that regulates trafficking of additional types of bone-marrow-derived stem and progenitor cells, such as endothelial progenitor cells [10, 39] and mesenchymal stem cells [7, 40], to ischemic/inflamed tissue. In accordance, local delivery of SDF-1 can enhance endothelial progenitor cell recruitment and neovascularization [41]. Several examples illustrate the contribution of bone-marrow-derived endothelial progenitor cells to improve cardiac function [10, 26, 41] and to enhance angiogenesis and neovascularization in several ischemic tissue models [42]. Of importance, inflammatory pathways in the injured organs also activate the recruitment of mature bone-marrow-derived cell types, which participate in mechanisms of tissue defense and repair as well. In addition, more differentiated bone-marrow-derived mature cells have been shown to establish perivascular niches prior to endothelial cell positioning and retention. Bone marrow recruitment of myeloid cells into injured heart and their retention in close proximity to angiogenic vessels is mediated by VEGF-induced expression of SDF-1 in activated perivascular myofibroblasts [43]. Moreover, it was shown that the homing of bone-marrow-derived hematopoietic c-kit$^+$ progenitor cells was accompanied by the recruitment of bone-marrow-derived mature natural killer cells in response to inflammation-secreted stress signals, which contributed to cardiac survival and repair after myocardial infarction [44]. However, in patients suffering from chronic ischemic coronary heart disease (ICMP), the ability of circulating endothelial progenitor cells to contribute to the neovascularization of the continuously inflamed heart is impaired with respect to number and functional activity. Note also that bone-marrow-derived mononuclear cells, isolated from patients with ICMP, have a significantly reduced migratory potential to a gradient of SDF-1 or VEGF, with reduced progenitor colony-forming activity *in vitro* and reduced neovascularization capacity *in vivo*, despite a similar content of hematopoietic progenitor cells, which would limit their therapeutic potential

for clinical cell therapy [45]. These results suggest that factors such as SDF-1, continuously secreted by the ischemic hearts of ICMP patients, can effectively reach the bone marrow, leading to desensitization and inhibition of endothelial and hematopoietic progenitors' migration and proliferation/differentiation.

Increased levels of SDF-1 in injured tissues are accompanied by modulations in the levels of additional factors, which actively participate in regulation of SDF-1 availability and function as well as in homing cell navigation and retention. Deficiency in nitric oxide synthase results in elevated SDF-1 levels in ischemic artery in a murine model, which is accompanied by increased numbers of circulating $Sca1^{+}c\text{-}Kit^{+}Lin^{-}$ stem cells [46]. Since this ligand also induces secretion of matrix metalloproteinases (MMPs), such as MMP2/9, SDF-1 elevation in damaged organs is also accompanied by an increase in and activation of various MMPs, which are involved in matrix degradation in the context of motility and *in vivo* migration of normal and malignant progenitor cells [3, 47].

Conclusion

In summary (schematically illustrated in Figure 1.1), dynamic SDF-1 and CXCR4 interactions regulate immature and mature bone-marrow-derived cell egress/mobilization in response to stress signals as well as their homing into injured organs aimed at tissue defense and repair mechanisms.

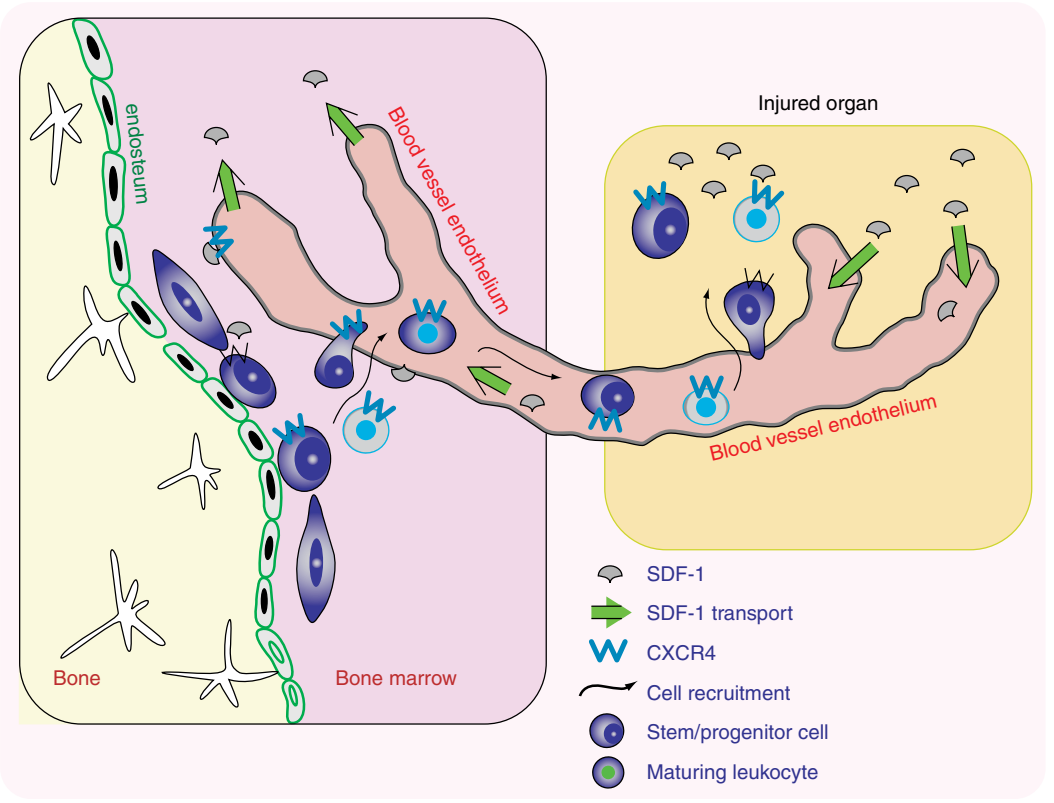


Figure 1.1 Immature and maturing cell recruitment mediated by SDF-1 transport: a model for communication between the injured organ and the bone marrow. Organ injury induces increased local production of SDF-1. Endothelial cells of the blood vessels translocate SDF-1 from the damaged tissue via the circulation into the bone marrow in a CXCR4-dependent manner. Presentation of the translocated SDF-1 by bone marrow endothelial and other stromal cells recruits CXCR4-expressing immature progenitors and stem cells as well as maturing leukocytes to the injured organ as part of host defense and organ repair.

Recent findings illustrate the participation of CXCR4 expressed by endothelial and other stromal cells in the bone marrow, spleen, and other organs, in regulating homing and retention of hematopoietic progenitors upon uptake and presentation of circulating functional SDF-1, leading to mobilization and recruitment of immature hematopoietic and endothelial progenitors to injured organs. These results suggest that CXCR4 expressed by stromal and endothelial cells actively participates in regulation of this mutual organ crosstalk during homeostasis and organ injury/damage. In cooperation with the bone marrow reservoir of hematopoietic and endothelial cells, $CXCR4^+$ progenitors and maturing cells with migration, proliferation, neovascularization, and defense potential participate in organ–bone marrow communication as part of host defense and repair mechanism. Taken together, these findings deepen our understanding of the significance of SDF-1 modulations in the circulation, bone marrow, and damaged organs, which accompany many pathological conditions and may contribute to the creation of improved clinical protocols.

References

1 Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol 2002; 30(9):973–981.

2 Stumm RK, Rummel J, Junker V, et al. A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. J Neurosci 2002; 22:5865–5878.

3 Kollet O, Shivtiel S, Chen YQ, et al. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J Clin Invest 2003; 112(2):160–169.

4 Assmus B, Honold J, Schachinger V, et al. Transcoronary transplantation of progenitor cells after myocardial infarction. *N Engl J Med* 2006; 355(12):1222–1232.

5 Schachinger V, Erbs S, Elsasser A, et al for the REPAIR-AMI Investigators: Intracoronary bone marrow-derived progenitor cells in a acute myocardial infarction. *N Engl J Med* 2006; 355(12):1210–1221.

6 Peled A, Petit I, Kollet O, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999; 283:845–848.

7 Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418(6893):41–49.

8 Hattori K, Dias S, Heissig B, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001; 193(9):1005–1014.

9 Boyle AJ, Schulman SP, Hare JM: Stem cell therapy for cardiac repair. Ready for the next step. *Circulation* 2006; 114:339–352.

10 Ceradini DJ, Kulkarni AR, Callaghan MJ, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004; 10:858–864.

11 Anversa P, Leri A, Kajstura J: Cardiac regeneration. *J Am Coll Cardiol* 2006; 47:1769–1776.

12 Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 2004; 95(4):343–353.

13 Petit I, Szyper-Kravitz M, Nagler A, et al. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat Immunol 2002; 3(7):687–694.

14 Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001; 103:2885–2890.

15 Wojakowski W, Tendera M. Mobilization of bone marrow-derived progenitor cells in acute coronary syndromes. Folia Histochem Cytobiol 2005; 43:229–232.

16 Aiuti A, Webb IJ, Bleul C, et al. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 1997; 185(1):111–120.

17 Ponomaryov T, Peled A, Petit I, et al. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J Clin Invest 2000; 106:1331–1339.

18 Imai K, Kobayashi M, Wang J, et al. Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitor cells to bone marrow. Br J Haematol 1999; 106(4):905–911.

19 Dar A, Goichberg P, Shinder V, et al. Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. Nat Immunol 2005; 6(10):1038–1046.

20 Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003; 425:836–841.

21 Sipkins DA, Wei X, Wu JW, et al. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 2005; 5(7044):969–973.

22 Pablos JL, Amara A, Bouloc A, et al. Stromal-cell derived factor is expressed by dendritic cells and endothelium in human skin. Am J Pathol 1999; 155(5):1577–1586.

23 Zou YR, Kottmann AH, Kuroda M, et al. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 1998; 393(6685):595–599.

24 Wojakowski W, Tendera M, Michalowska A, et al. Mobilization of CD34/CXCR4+, CD34/CD117+, c-met+ stem cells, and mononuclear cells expressing early cardiac, muscle, and endothelial markers into peripheral blood in patients with acute myocardial infarction. Circulation 2004; 110(20):3213–3220.

25 Lapidot T, Dar A, Kollet O. How do stem cells find their way home? Blood 2005; 106(6):1901–1910.

26 Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003; 362:697–703.

27 Gupta SK, Pillarisetti K. Cutting edge: CXCR4-Lo: molecular cloning and functional expression of a novel human CXCR4 splice variant. J Immunol 1999; 163(5):2368–2372.

28 Heissig B, Werb Z, Rafii S, Hattori K. Role of c-kit/Kit ligand signaling in regulating vasculogenesis. Thromb Haemost 2003; 90:570–576.

29 Tachibana K, Hirota S, Iizasa H, et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 1998; 393(6685):591–594.

30 Salcedo R, Wasserman K, Young HA, et al. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1alpha. Am J Pathol 1999; 154:1125–1135.

31 Hattori K, Heissig B, Tashiro K, et al. Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood 2001; 97(11):3354–3360.

32 Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995; 1:27–31.

33 Sweeney EA, Lortat-Jacob H, Priestley GV, et al. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells. Blood 2002; 99:44–51.

34 Shen H, Cheng T, Olszak I, et al. CXCR-4 desensitization is associated with tissue localization of hemopoietic progenitor cells. J Immunol 2001; 166:5027–5033.

35 Kollet O, Samstein R, Spiegel A, et al. Osteoclasts are involved in stem cell mobilization: cleavage of SDF-1 by cathepsin K. Blood 2004; 104:364A–364A.

36 Abkowitz JL, Robinson AE, Kale S, et al. Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. Blood 2003; 102(4):1249–1253.

37 Tuma PL, Hubbard AL. Transcytosis: crossing cellular barriers. Physiol Rev 2003; 83:871–932.

38 Kortesidis A, Zannettino A, Isenmann S, et al. Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. Blood 2005; 105:3793–3801.

39 Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002; 109(5):625–637.

40 Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol 2005; 23:845–856.

41 Kawamoto A, Tkebuchava T, Yamaguchi J, et al. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107:461–468.

42 De Falco E, Porcelli D, Torella AR, et al. SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. Blood 2004; 104:3472–3482.

43 Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 2005; 124(1):175–189.

44 Ayach BB, Yoshimitsu M, Dawood F, et al. Stem cell factor receptor induces progenitor and natural killer cell-mediated cardiac survival and repair after myocardial infarction. Proc Natl Acad Sci USA 2006; 103:2304–2309.

45 Heeschen C, Lehmann R, Honold J, et al. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation 2004; 109:1615–1622.

46 Zhang LN, Wilson DW, da Cunha V, et al. Endothelial NO synthase deficiency promotes smooth muscle progenitor cells in association with upregulation of stromal cell-derived factor-1{alpha} in a mouse model of carotid artery ligation. Arterioscler Thromb Vasc Biol 2006; 26(4):765–772.

47 Tessone A, Feinberg MS, Barbash IM, et al. Effect of matrix metalloproteinase inhibition by doxycycline on myocardial healing and remodeling after myocardial infarction. Cardiovasc Drugs Ther 2005; 19:383–390.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

2

CHAPTER 2

Adult stem cell plasticity: lineage potential on a continuum

Peter J. Quesenberry, MD, *Gerald Colvin,* DO, *& Mehrdad Abedi,* MD

Adult marrow stem cells represent the most extensively studied stem cell population, and the hematopoietic system the most completely studied renewal/differentiation system. Early studies showed that differentiation into blood cells at the morphological level was hierarchical [1–5]. Myeloblasts sequentially differentiated into mature granulocytes and erythroblasts into mature erythrocytes. These studies combined time course evaluations of murine marrow recovery from cytotoxic insults, with tritium labeling of cells to define proliferative and nonproliferative maturing marrow populations, and established the hierarchical nature of hematopoiesis when relatively mature elements of the system were considered.

The description of the colony-forming unit spleen (CFU-s), the first clonal stem cell assay [6], immediately suggested a stem cell to end cell differentiation hierarchy. The CFU-s had proliferative potential and could differentiate into all myeloid lineages. Subsequent descriptions of granulocyte-macrophage progenitors assayed in clonal *in vitro* cultures [7–8] indicated the probable existence of a hierarchy of pluripotent stem cells (CFU-s) differentiating to progenitors (CFU-GM, colony-forming unit granulocyte-macrophage) to morphologically recognizable myeloid cells. Subsequently, there also followed descriptions of erythroid and megakaryocyte progenitors, which could all be placed in a very logical hierarchical system [9–17]. Over time, a large number of progenitors, assayed *in vitro*, with virtually all types of lineage combinations, were described, including single lineage progenitors. When studies on *in vivo* assays for long and short-term repopulating cells were reported, the significance of the CFU-s was debated, but the probability of a hierarchical system from a primitive renewal stem cell, to a multilineage progenitor, to a single lineage progenitor, to differentiated cells was strengthened. The hierarchical nature of the system was further supported by *in vitro* studies showing the decline of long-term engraftable stem cells with expansion of progenitor populations, and by studies indicating a hierarchical transcription factor regulation of hematopoiesis [18–20].

However, from the very beginning there was evidence that the system at the progenitor stem cell level was not a neat orderly hierarchy. Till et al. [21] noted the heterogeneity of renewal capacity of CFU-s and proposed that the system was basically stochastic. They compared these populations to radioactive nuclei, where the decay rate of the population of nuclei was highly predictable, but that of individual nucleus totally heterogeneous and unpredictable. The continued observations describing the heterogeneity of marrow stem cells should have urged caution to many investigators who were proposing a classical hierarchical model. In recent studies on perhaps the most purified cycle synchronized marrow stem cell population, the lineage negative rhodamine low Hoechst (LRH) stem cell, total heterogeneity was found at isolation or at specific points in the cell cycle when these cells were cultured on a single cell basis in cytokines and analyzed regarding gross colony morphology and the lineage makeup of the colonies (GA

Colvin, PJ Quesenberry, unpublished observation, 2006).

Work by Suda et al. [22] indicated that a strict hierarchical system was very unlikely. They cultured primitive stem/progenitor cells and separated daughter cells from an early proliferative unit and then followed differentiation of the daughter cells under permissive cytokine conditions. In a hierarchical system, one would expect that each daughter cell would give the same differentiation outcome when placed under similar cytokine conditions. However, in approximately 20% of the cases, the differentiation outcomes did not fit into an ordered hierarchy. Thus, one colony might have erythroid and megakaryocyte elements, and the other sister colony, granulocyte and macrophage components. Recently, Takano et al. [23], studying asymmetric division and lineage commitment at the level of the murine hematopoietic stem cell, presented strong data arguing against a purely hierarchical system and against the recently proposed concept regarding a common lymphoid and common myeloid stem cell as part of a hierarchy.

In this chapter, we will provide the arguments for and against the biologic phenomenon of stem cell plasticity.

Stem cell plasticity

Stem cell plasticity, of course, directly relates to the concepts of adult marrow stem cell heterogeneity and hierarchy, or the lack thereof. It also expands these concepts into nonhematopoietic cells and tissues. Viewed simply, recent data in this area would indicate that marrow stem cells have more and broader differentiation potential than previously realized. As will be discussed, the temporal plasticity of the stem cell system indicates great flexibility and further expands its potential. In order to address the full potential of the adult marrow stem cell system, we first need to address the controversies that have arisen around adult marrow stem cell plasticity.

First, what are the observations that demonstrate stem cell plasticity does exist? Lagasse and colleagues [24], using the fumarylacetoacetate hyroxylase (FAH) deficient mouse, which without appropriate drug treatment 2-(2-nitro-4-trifluoromethyl benzoyl)-cyclohexane-1,3-done (NTBC) dies of fatal tyrosinemic liver disease, showed that purified marrow stem cells could produce robust numbers of hepatocytes and cure the mice. Additional studies confirmed that marrow cells can produce nonhematopoietic tissue cells in liver, skeletal muscle, and lung [24–34] (J Aliotta, M Dooner, J Pimentel, PJ Quesenberry, unpublished observation, 2004), albeit tissue function has been more difficult to approach. These phenomena occur in other tissues such as the heart, skin, bone, pancreas, cartilage, gastrointestinal tract, kidney, and brain and have, in general, given a lower percentage of total tissue cells, but are still demonstrable [35–55]. Cardiac studies, which are extensively dealt with in this book, have been relatively controversial and represent the only plasticity studies where competent investigators have carried out similar studies with divergent results [42, 43]. Some of the other "negative" studies will be discussed below. However, when examined altogether, a large number of studies have shown that marrow cells can produce or be involved in the creation of nonhematopoietic tissues in a variety of tissues.

The mantra for adult marrow stem cell plasticity to be considered important or real is that one has to do the work clonally, the phenomenon has to be robust, and cell fusion must not be involved [56–58]. In addition, major criticisms have been put forward as to the validity of donor cell identification of nonhematopoietic tissue cells in various organs. It has also been stressed that transdifferentiation must be established. This debate has been driven, in part, by negative studies purporting not to reproduce many of the positive results shown by others. It is worth addressing each component of this debate, which has been previously characterized as "Ignoratio Elenchi" [59].

Cell identification

Perhaps the most important issue is the validity of cell identification. Concerns have been expressed about the validity of autofluorescence, with green fluorescent protein (GFP) studies, and about a false reading of cellular overlap. In general, our own reading of these studies is that the more prominent ones have been carefully carried out, with full attention given to a rigorous demonstration of the donor origin of tissue cells, having been identified with tissue-specific markers. In contrast, the negative studies have

been sparse in detail and do not appear to have been carried out with as much rigour. Overlapping cells and autofluorescence in the lung have been cited as major problems causing false positive results, indicating marrow to lung conversions [60, 61]. The same investigators failed to find any examples of conversions despite the large number of studies showing that they exist. In detailed studies in our laboratory, using a GFP+ marrow transplant system and anti-GFP antibodies to specifically mark GFP, we found no evidence of confounding autofluorescence. In our studies, all slides are read with a double-band-pass filter. We also carried out extensive deconvolution and confocal studies to determine whether the presence of GFP positive CD45− or cytokeratin+ cells were confounded by the presence of adjacent cells. In cells that we determined had a nucleus (DAPI staining), which were GFP+ and either CD45− or cytokeratin+, we are yet to find evidence of overlapping cells. Finally, in studies that failed to detect nonhematopoietic donor cells, these investigators carried out an intraperitoneal transplant of newborn mice, which is not a standard transplant model and not an approach used by others. A transgenic surfactant-promoter model was used and no positive cells were found. However, this model was never validated and only a small percentage of the transgenic cells expressed this promoter.

Transdifferentiation

The concept of transdifferentiation and the importance of showing that the phenomenon represents transdifferentiation is a red herring or Ignoratio Elenchi. There is no a priori reason to insist that the observation that donor marrow cells can assume a nonhematopoietic cell phenotype has to be ascribed to transdifferentiation, but that is what some would insist upon. In fact, as far as we are aware, transdifferentiation has never been absolutely established in any system, including that of the newt or skate. In these cases, differentiation from small populations of resident stem cells has never been ruled out. In fact, it would seem likely that the major mechanism underlying the observed plasticity is probably differentiation from relatively small numbers of functionally primed stem cells with subsequent selection. This, of course, remains to be established.

Clonality

This is another red herring. As described above, primitive marrow stem cells are very heterogeneous. Therefore, single-cell studies tell you about the heterogeneity. They are unnecessary for plasticity work. The appropriate studies for stem cell plasticity should be on defined populations of cells. Single-cell studies can then be carried to establish the nature of the heterogeneity.

Robustness

This is relatively meaningless. A single stem cell may lead to a "robust" repopulation under repetitive injury conditions, for example, as existed in the FAH studies. Alterations in tissue function should be considered the end game of this research, not a criticism of the initial studies on the phenomena. It is probably worth mentioning that the work with embryonic stem cells has led to almost no demonstrations of *in vivo* function and those few are clouded by the probability that they are demonstrating the potential of neoplastic cells.

Fusion

In many reports, there has been a significant replacement of host tissue cells with cells derived from donor marrow cells, either by fusion or differentiation, with levels ranging from 0.2–35% for replacement of skeletal muscle or lung tissue [62–64]. There have been a fairly wide range of values for percent donor cells acquiring a nonhematopoietic phenotype in different tissues. A noninclusive list is outlined for skeletal muscle, lung, and liver in Tables 1 and 2.

In studies on the capacity of GFP+ marrow cells to differentiate into or fuse *in vivo* with skeletal muscle and lung, we have found many variables affecting the outcome. It is the differences in specific methodology and application of these variables that probably explain the variance of results with these plasticity studies. We have found, when studying either marrow to lung or skeletal muscle, that the percentage of donor-derived cells is influenced by many variables, most prominently the nature and extent of the injury to the tissue under consideration [29, 33]. Without any injury, at least in lung or skeletal muscle, one sees very rare to no donor marrow cells with the phenotype of nonhematopoietic

Table 2.1 Marrow to lung and skeletal muscle conversions.

Tissue	*Injury*	*Donor cells*	*Conversion result (%)*	*Ref.*
Skeletal Muscle	Radiation and exercise	GFP marrow	3.5	[25]
	TBI/mdx	Spleen marrow	0.2	[26]
	TBI/mdx	Marrow side population	1–10	[27]
	TBI/cardiotoxin	GFP marrow	1–2	[28]
	G-CSF mobilization and direct injection of lineage negative marrow cells		up to12.5	[29]
	α-Sarcoglycan null dystrophic mice	Blood vessel-associated fetal stem cells	50	[62]
Lung	600 cGy	GFP marrow, mononuclear cells or side population	1–7	[31]
	Radiation	Rosa MAPC	2–10	[50]
	1050 cGy	Fr25/Lin−	20	[34]
	900 cGy, cardiotoxin or bleomycin G-CSF mobilization	GFP marrow	35	[33]

this table is hard to read in its current form. Check that injury column correctly matches up with donor cell column. NEED ABBREVIATIONS SPELLED OUT GFP = green fluorescent protein; MAPC = multipotent adult progenitor cells; TBI/mdx = total body irradiation/muscular dystrophy; G-CSF = granulocyte colony-stimulating factor.

tissue cells. Variables that influence donor cell replacement of host tissue cells are outlined in Table 3.

The initial studies of Lagasse and colleagues [24], which showed a cure of FAH- mice, were said to be due to fusion. So why is fusion a limiting consideration? Fusion is simply one of many means to an end, and, in fact, it is model dependent. In studies of liver cell repopulation, fusion is present in some

Table 2.2 Marrow to liver conversions.

Tissue	*Injury*	*Cell source*	*Conversion rate*	*Ref.*
Liver	FAH− mouse	Lin−Sca-1+c-kit+	30–50%	[24]
	NTBC withdrawal	Thy−1+ marrow cells Rosa	liver mass	
	Irradiated	Rosa26 MAPC marrow cells	5–8%	[50]
	Irradiation	GFP marrow	Small clusters hepatocytes, 0.07%	[30]
	Irradiation, CCL4 or Retrosine	GFP marrow	Hepatocytes present	[27]
	No injury, Fetal sheep	USSC cord blood cell	20%	[63]*
	Radiation	Cre/lox Z/EG Mouse marrow	0.05–0.1%	[64]*

*Fusion ruled out as conversion mechanism.
Likewise, be sure injury and cell source columns match up correctly.
FAH = fumaryl acetoacetate hydroxylase; NTBC = 2-(2-nitro-4-trifluoromethyl benzoyl)-cyclohexane-1,3-done; MAPC = multipotent adult progenitor cells; GFP = green fluorescent protein; USSC = unrestricted somatic stem cells.

Table 2.3 Variables influencing derivation of host tissue cells from donor marrow.

Tissue injury—type, extent, and location
Cell dose
Timing of injury and infusion
Marrow mobilization
Stem cell population
Functional (cell cycle) state of marrow cell
Route of cell infusion—arterial, venous, or into tissue
Transplant regimen (dose and location of irradiation)

models and not the mechanism of repopulation in others [65–70]. This holds true for other reports of fusion as outlined in Tables 4 and 5.

Negative reports

The debate has been strongly influenced by negative reports relating to stem cell plasticity. These reports have claimed an inability to reproduce the work of others, but in fact, in all but one notable case, the studies have not attempted to reproduce the initial work. In one of the most highlighted papers, Wagers et al. [71] said that they had failed to reproduce the studies of Krause et al. [34]. This statement was essentially retracted later in a letter to *Science* [72]. In that letter, Dr. Wagers and colleagues said, "Thus, our data are not directly comparable to those of Krause et al. and do not implicitly refute their observations." In the not too distant past, a failure to reproduce someone else's work generally meant that you had not done the studies correctly. We think this is still the case, but somehow the negative studies have been given more credence than the positive studies, and some very marginal negative studies have been published in our "top" journals. In any case, only the Murry paper [43] on cardiac plasticity appeared to truly try to reproduce the original work of Orlic et al. [42] and failed to confirm their observations.

These red herrings comprise the debate's mantra. In our recent perspective in *Science*, the editors removed the following cartoon (see Figure 2.1) as "nonconciliatory." We think it summarizes things nicely.

The continuum model

The above describes the heterogeneity of marrow stem cells within the hematopoietic system and, as

Table 2.4 Fusion demonstrated in converted cells.

Tissue/cell	*Model/detection*	*Ref.*
Hepatocyte	Fah+/+ from Fancc−/− into Fah −/− with NTBC withdrawal, 50% conversion rate. Purified repopulating cells were heterozygous Fah+/+ and Fanc−/−	[24]
Hepatocyte	Fah+/+ from Rosa female marrow into male Fah−/−. Cytogenetic analysis of LacZ+ marrow derived hepatocytes—most with Y chromosome. Karyotypes Fah+/+ 80XXXY or 120 XXXXYY.	[65]
Purkinje neuron	GFP to adult mice and both donor and host nuclei found; the Purkinje neurons were stable heterokaryons.	[66]
Purkinje neuron, Cardiomyocyte, Hepatocyte	Used Cre/loz recombinase system to show that in marrow transplanted mice all detectable contributions of marrow to nonhematopoietic cell types arose through cell fusion.	[67]
Skeletal	Murine cardiotoxin injury model male to female, female to male or Rosa B-galactosidase to GFP muscle fibers show both donor and recipient phenotypes. However, mononuclear satellite cells with donor markers suggest conversion to satellite cells occurs without fusion.	[28, 29]

NTBC = 2-(2-nitro-4-trifluoromethyl benzoyl)-cyclohexane-1,3-done; GFP = green fluorescent protein.

Table 2.5 Conversions without fusion.

Tissue/cell	*Model/detection*	*Ref.*
Pancreas	Rosa–stop lox and GFP female hosts transplanted with insulin-dependent Cre-male marrow. No GFP+ donor cells in islets.	[46]
Hepatocyte	Human cord blood to irradiated NOD/SCID mouse. Human hepatocytes with positive protein and chromosome markers, no mouse chromosomes. Conversion rate 1–2%.	[68]
Hepatocyte	Human cord blood (USSC) into fetal sheep without injury, 20% conversion rate. Microdissected human hepatocytes had only human protein or PCR product.	[63]
Endothelial	c-kit+ ,Sca-1+, Lin− into irradiated mouse; Donor endothelia in portal vein, normal ploidy; also cord blood to mouse with new blood vessel formation in the eye—no fusion.	[69]
Renal mesangial cells	Male GFP marrow to male mice resulted in numerous GFP+ mesangial cells; none had >1 Y chromosome.	[70]
Epithelial cells into lungs, skin, and liver	Cre/lox recombinase system. Transplant Z/EG marrow Cre expressing mice. No mice expressed GFP, indicating that fusion had not occurred.	[64]
Skeletal	Converted mononucleated satellite cells precede muscle fiber fusion.	[25]

GFP = green fluorescent protein; NOD-SCID = non-obese diabetic severe immune deficiency; USSC = unrestricted somatic stem cells.

part of that inherent heterogeneity, their capacity to differentiate into a broader range of cell types and tissues other than just hematopoietic. There is further elasticity of this system represented by shifts in the phenotypes of marrow stem cells over time, apparently linked to the phase of the cell cycle.

Any cell, of necessity, changes phenotype with cell cycle transit, probably due to changes in chromatin/histone configuration [73–76] and the availability of various promoter regions for interactions with transcription factors. The stem cell has generally been felt to be dormant or in G0. However, it

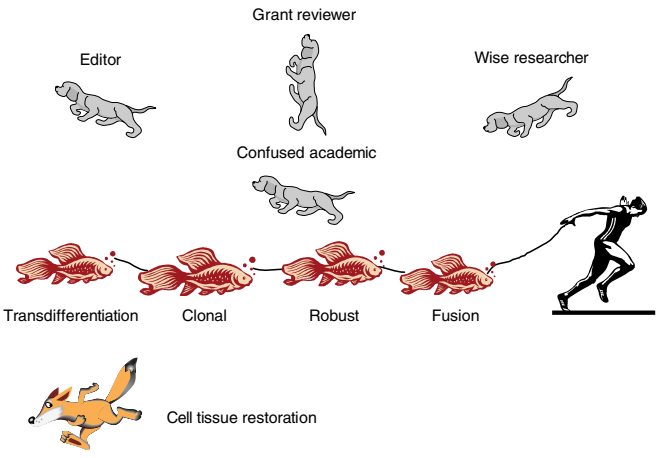


Figure 2.1 Red herrings in stem cell plasticity. In fox hunting, drawing red herrings across the path of the escaping fox masks the scent of the fox and confuses the hound dogs. Here, we show the fox (stem cell tissue restoration) escaping.

has been shown that virtually every stem cell type, with one exception, when evaluated as to cell cycle status by such methods as tritiated thymidine labeling or suicide, hydroxyurea suicide, or propidium labeling and flow analysis, has a small percentage of their population in S phase, running at a rate of 3–5% [77–83]. This suggests that they are all probably in slow cycle or the entering and exiting cycle. The one exception to this is the LRH stem cell [84], which is separated on the basis of dormancy and which generally has no cells in the S phase. However, this cell is easily induced into the cell cycle by *in vivo* engraftment or *in vitro* exposure to cytokines [85, 86]. Bradford and colleagues [87] used the LRH cell to show that the most primitive stem cells were cycling *in vivo*. They fed mice with BrdU via drinking water, sacrificed them at different points in the feeding schedule, isolated the LRH cells, and, using anti-BrdU labeling, determined the percent of LRH cells that were labeled. The latter, of course, would indicate the percent of LRH cells that had entered S phase during the feeding schedule. They found that 60% of LRH cells were labeled by 4 weeks. We confirmed these results [88], while Cheshire and colleagues [82], using a different mouse strain and stem cell separation, but the same basic model, also confirmed the Bradford results, but with more rapid kinetics. Thus, stem cells are a cycling cell population. This is important for our understanding of the significance of studies, which we will describe below, showing that LRH cells demonstrate reversible changes in phenotype with cycle transit.

We have established that murine marrow stem cells show marked changes in short- and long-term engraftment as they march through a cytokine stimulated cell cycle [89, 90]. The cell cycle progression of LRH murine stem cells has been mapped through cell cycle transit under stimulation with two different cytokine cocktails: either interleukin 3 (IL-3), IL-11, IL-6, and steel factor [86] or thrombopoietin, FLT3L, and steel factor. While the kinetics of cell cycle passage differed between the cytokine regimes in each case, engraftment capacity was markedly decreased in late S phase/early G2 and, subsequently, recovered in the next cycle. We demonstrated that there were marked and reversible shifts in engraftment capacity when competitive engraftment was assessed in a lethally irradiated mouse transplant model, and these shifts could occur over 2–4-hour time intervals. This was found whether whole marrow or purified LRH were tested at different points in a cytokine culture. When mapping the cell cycle of LRH cells under stimulation with IL-3, IL-6, IL-11, and steel factor, there were essentially no cells in S phase at isolation, there was a prolonged initial G0/G1 phase followed by entry into S phase at about 18–20 hours and entry into mitosis at about 36–38 hours. Subsequently, the LRH cells exhibited a tight synchrony through five additional cycles (the cycle lengths were 12 hours). Analysis of these cycles Lin-Sca-1+ cells showed that 15–20% of these cells were in S phase at the time of isolation and that completion of the first cell cycle was longer than that with the other cytokine cocktail.

The reason for the changes in engraftment probably was based on changes of marrow homing. We established a homing assay in which 200,000–250,000 Lin-Sca-1+ marrow stem cells were labeled with CFSE, infused, and then recovered 3 hours later from the marrow. Using this assay, we determined that homing was markedly depressed at 48 hours, a time when engraftment was also reversibly depressed [91]. The homing defect was probably due to cell-cycle-related changes in multiple homing proteins, the most prominent being VLA-4 [92, 93] and CXCR.

Further work indicated that progenitor levels also fluctuated reversibly with the cell cycle and that these levels correlated inversely with the levels of engraftable stem cells; we termed these changes stem cell/progenitor cell inversions [94]. These changes suggested that the progenitors as defined by various *in vitro* assays might simply be functional variants of the engraftable stem cells and not a differentiated progeny of them.

In addition, we have used gene display (a nonbiased approach) of purified LRH stem cells and of lineage positive cells from the same starting population and defined a total of 637 stem-cell-specific mRNAs [90]. Since 411 of these were unknowns, it was difficult to come to mechanistic conclusions, but there was a dramatic shift in global gene expression at 48 hours of culture with IL-3, IL-6, IL-11, and steel factor—a time at which both homing and engraftment were markedly depressed. In addition, using real-time PCR, we have shown major shifts of both hematopoietic transcription factors and hematopoietic cell surface markers with

cycle transit. These shifts emphasized the variable and continually changing nature of the stem cell, associated with cell cycle transit.

These stem cell phenotypic shifts are consistent with the continually changing opportunities for transcriptional activation that would occur with cycle transit. If this is the case, then differentiation potential should also be changing and that potential could hold for both hematopoietic and nonhematopoietic stem cells. We tested this proposition by evaluating the potential for LRH cells to differentiate in response to the cytokines granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF) and steel factor, while transiting the cycle under stimulation with thrombopoietin, FLT3L, and steel factor (GA Colvin, PJ Quesenberry, unpublished observation, 2006), and we found that there were points in the cell cycle in which granulocyte or megakaryocyte differentiation was favored. We referred to these as hot spots. The megakaryocyte hot spot occurred at the G1/S phase interface, while the granulocyte hot spot occurred at mid S phase. There were marked fluctuations of macrophage differentiation in individual experiments, but these were temporally inconsistent. Overall, these data indicated that differentiation into hematopoietic cells showed lability with cell cycle passage. The plasticity of the marrow stem cell tied to cell cycle transit within the hematopoietic system is extensive and has led to a continuum theory of hematopoietic regulation (see Table 6).

The above list is not inclusive of all time related fluctuations in stem cell phenotype. The expression of membrane extensions, which we have called proteopods [95], vary over time, and we have demonstrated circadian rhythms for both engraftable stem cells and progenitors—out of phase with each other [96].

Table 2.6 The cell-cycle-related shifting phenotype of the marrow stem cell: the continuum.

Characteristics that show cell cycle related alterations
Engraftment—long- and short-term
Homing to marrow
Adhesion protein expression
Cytokine receptor expression
Cell surface marker and transcription factor expression
Progenitor numbers and type
Hematopoietic differentiation

Given our discussion of stem cell plasticity with regard to probable differentiation into nonhematopoietic cells, it seemed likely that such differentiation would also occur on a continuum. We have used a model of GFP+ marrow transplanted into lethally irradiated mice to assess whether differentiation into lung cells varied at different points in a cytokine stimulated cell cycle transit. We have, in fact, found marked cycle-related fluctuations in marrow donor lung cell derived GFP+ CD45− or GFP+ cytokeratin+ cells, showing that marrow stem cell production of nonhematopoietic cells also occurs on a continuum. This is, in essence, plasticity squared.

Globally, it would appear that marrow stem cells have a continually changing capacity to produce different cell types, which will be expressed in the appropriate environment. This is consistent with both the phase-space model of Kirkland [97] and with the "Within- tissue" model of Roeder and Loeffler [98].

As yet, the mechanisms underlying the observed plasticity of marrow stem cells remain to be defined. Certainly, under certain circumstances of tissue injury, fusion is an important component of the production of substantial numbers of nonhematopoietic cells by a donor marrow cell. However, this is model dependent and in the case of skeletal muscle, where fusion is a normal response to injury, work by several groups suggests that donor cell origin of muscle satellite cells is not based on fusion. There is no evidence of transdifferentiation, but simple differentiation from highly potent marrow stem cells placed in the right environment is a reasonable probability.

There have been intriguing reports of changes in marrow cell phenotype when a nonhematopoietic tissue is cocultured across a cell impermeable membrane from marrow cells [99]. This suggests a diffusible factor mediating cell change. In our laboratory, we have seen stem cells enter and exit stromal cells, providing another potential mechanism for transfer of information. The intriguing work by Ratajczak and colleagues [100] showing cell vesicles with high levels of mRNA as a possible vehicle for the transfer of mRNA has been confirmed in our

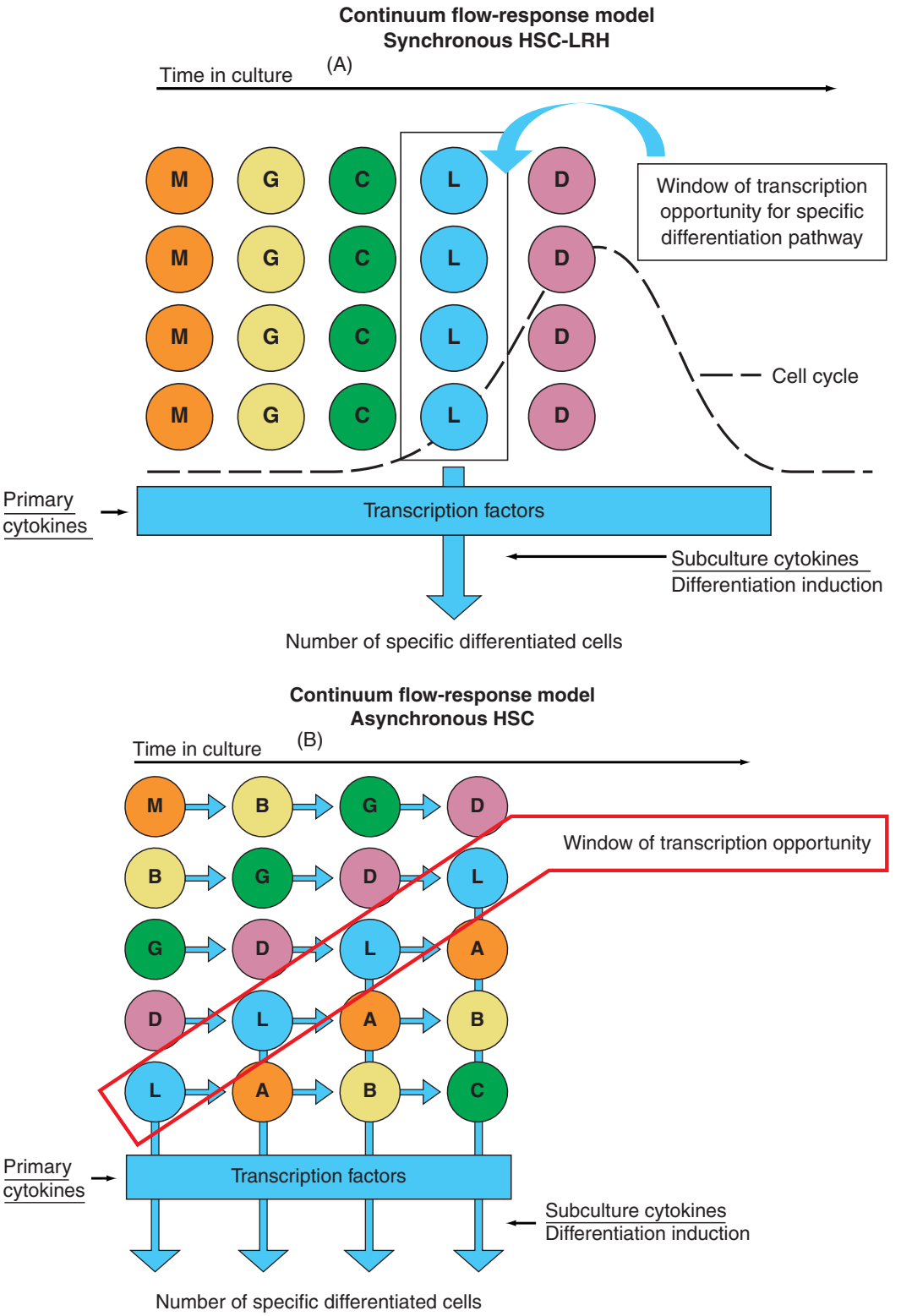


Figure 2.2 Continuum flow response model. (A) Synchronous HSC-LRH; (B) asynchronous HSC. HSC = hematopoietic stem cells; LRH = rhodamine low Hoechst stem cells

laboratory and provides another possible mechanism for epigenetic change.

Summary

A global model summarizing our cell cycle studies is presented in Figures 2A and 2B. Here, we speculate that as the stem cell progresses through the cell cycle, with alternate changes in chromatin–histone configurations, different promoter regions are accessible to their transcription factors. When induced transcription factors bind the appropriate promoter, terminal differentiating inducers can then act to direct a specific differentiation pathway, either hematopoietic or nonhematopoietic. Delivery of mRNA into the cell could also be a mediator of epigenetic change that could then be differentially expressed as the stem cell progresses through the cell cycle. Figure 2A presents the situation when synchronized stem cells are considered, such as in our model of cytokine-stimulated LRH cells. Figure 2B presents the same concepts in the context of nonsynchronized marrow stem cells.

Acknowledgments

The present studies were supported by Grant numbers R01 DK61858, R01 DK60090, R01 HL073749, R01 DK60084, KO8 HL72332, and KO8 DK64980. This publication was also made possible by NIH Grant number P20 RR 018757 from the COBRE Program of the National Center for Research Resources

References

1 Cronkite EP, Bond VP, Fleidner TM, Killmann SA. The use of tritiated thymidine in the study of haemopoietic cell proliferation. In Wolstenholme GEW, O'Connor M (eds): Ciba Foundation on Haemopoiesis. London: J and A Churchill Ltd, 70–92, 1960.

2 Cronkite EP, Vincent PC. Granulocytopoiesis. Ser Haematol 1969; 11:3–43.

3 Cronkite EP, Burlington H, Chanana AD, Joel DD, Reincke U, Stenes J. Concepts and observations on the regulation of granulopoiesis. In Baum SJ, Ledney GD (eds): Experimental Hematology Today. New York: Springer-Verlag, 41–48, 1977.

4 Fleidner TM, Cronkite EP, Killman SA, Bond VP. Granulocytopoiesis II. Emergence and pattern of labeling of neutrophilic granulocytes in human beings. Blood 1964; 24:683–700.

5 Cronkite EP. Hemopoietic stem cells: an analytic review of hemopoiesis. Pathobiol Annu 1975; 5:34–69.

6 Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 1961; 14:213–222.

7 Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. J Exp Biol Med Sci 1966; 44:287–300.

8 Pluznik DH, Sachs L. The cloning of normal "mast" cells in tissue culture. J Cell Comp Physiol 1965; 66:319–324.

9 McLeod DL, Shreeve MM, Axelrad AA Chromosome marker evidence for the bipotentiality of BF4-E. Blood 1980; 56(2):318–322.

10 McLeod DL, Shreeve MM, Axelrad AA. Improved plasma culture system for production of erythrocytic colonies in vitro: quantitative assay method for CFU-E. Blood 1974; 44:517–534.

11 Iscove NN, Siever F. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. Exp Hematol 1975; 3:32–43.

12 Nakeff A, Dicke KA, Van Noord MJ. Megakaryocytes in agar cultures of mouse bone marrow. Ser Haematol 1975; 8:4–21.

13 Metcalf D, MacDonald HR, Odartchenko N, et al. Growth of mouse megakaryocyte colonies in vitro. Proc Natl Acad Sci USA 1975; 72:1744–1748.

14 Heath DS, Axelrad AA, Mc Leod DL, Shreeve MM: Separation of the erythropoietin-responsive progenitors ßFu-E and CFu-E in mouse bone marrow by unit gravity sedimentation. Blood 1976; 47(5):777–792.

15 Gregory CJ. Erythropoietin sensitivity as a differentiation marker in the hemopoietic system: studies of three erythropoietic colony responses in culture. J Cell Physiol 1976; 89:289–301.

16 Long MW, Gragowski LL, Heffner CH, Boxer LA. Phorbol diesters stimulate the development of an early murine progenitor cell. The burst-forming unit-megakaryocyte. J Clin Invest 1985; 76:431–438.

17 Briddell RA, Brandt JE, Straneva JE, Srour EF, Hoffman R. Characterization of the human burst-forming unit-megakaryocyte. Blood 1989; 74:145–151.

18 Shivdasani RA, Orkin SH. The transcriptional control of hematopoiesis. Blood 1996; 87:4025–4039.

19 Pevny L, Simon CM, Robertson E, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 1991; 349:257–260.

20 Cantor AB, Orkin SH. Hematopoietic development: a balancing act. Curr Opin Genet Dev 2001; 11:513–519.

21 Till JE, McCuloch EA, Siminovitch L. A stochastic model of stem cell proliferation, based on the growth of spleen

colony-forming cells. Proc Natl Acad Sci USA 1964; 51:29–36.

22 Suda T, Suda J, Ogawa M. Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. Proc Natl Acad Sci USA 1984; 81:2520–2524.

23 Takano H, Ema H, Sudo K, Nakauchi H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cells and granddaughter cell pairs. J Exp Med 2004; 199:295–302.

24 Lagasse E, Connors H, Al-Dhalimy M, et al. Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. Nat Med 2000; 6:1229–1234.

25 LaBarge MA, Blau HM. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell 2002; 111(4):589–601.

26 Bittner RE, Schofer C, Weipoltshammer K, et al. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. Anat Embryol (Berl) 1999; 199(5):391–396.

27 Gussoni E, Soneoka Y, Strickland CD, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 1999; 401(6751):390–394.

28 Abedi M, Greer DA, Colvin GA, et al. Tissue injury in marrow transdifferentiation. Blood Cells Mol Dis 2004; 32(1):42–46.

29 Abedi M, Greer DA, Colvin GA, et al. Robust conversion of marrow cells to skeletal muscle with formation of marrow-derived muscle cell colonies: a multifactorial process. Exp Hematol 2004; 32(5):426–434.

30 Ortiz LA, Gambelli F, McBride C, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci USA 2003; 100(14):8407–8411.

31 Abe S, Lauby G, Boyer C, Rennard S, Sharp J. Transplanted BM and BM side population cells contribute progeny to the lung and liver in irradiated mice. Cytotherapy 2003; 5(6):523–533.

32 Grove JE, Lutzko C, Priller J, et al. Marrow-derived cells as vehicles for delivery of gene therapy to pulmonary epithelium. Am J Respir Cell Mol Biol 2002; 27(6):645–651.

33 Aliotta J, Keaney P, Passero M, et al. Bone marrow production of lung cells: the impact of G-CSF, cardiotoxin, graded doses of irradiation and subpopulation phenotype. Exp Hematol 2006; 34:230–241.

34 Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001; 105(3):369–377.

35 Pereira R, Halford K, O'Hara M, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci USA 1995; 92:4857–4861.

36 Nilsson SK, Dooner MS, Weier HU, et al. Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. J Exp Med 1999; 189:729–734.

37 Badiavas EV, Falanga V. Treatment of chronic wounds with bone marrow-derived cells. Arch Dermatol 2003; 139(4):510–516.

38 Badiavas EV, Abedi M, Butmarc J, Falanga V, Quesenberry PJ. Participation of bone marrow derived cells in cutaneous wound healing. J Cell Physiol 2003; 196(2):245–250.

39 Kataoka K, Medina RJ, Kageyama T, et al. Participation of adult mouse bone marrow cells in reconstitution of skin. Am J Pathol 2003; 163(4):1227–1231.

40 Direkze NC, Forbes SJ, Brittan M, et al. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. Stem Cells 2003; 21(5):514–520.

41 Nygren JM, Jovinge S, Breitbach M, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004; 10(5):494–501.

42 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410(6829):701–705.

43 Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428(6983):664–668.

44 Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107(11):1395–1402.

45 Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling, and improves cardiac function. Nat Med 2001; 7(4):430–436.

46 Ianus A, Holz GG, Theise ND, Hussain MA. *In vivo* derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 2003; 111(6):843–850.

47 Hess D, Li L, Martin M, et al. Bone marrow-derived stem cells initiate pancreatic regeneration. Nat Biotechnol 2003; 21(7):763–770.

48 Hoi JB, Uchino H, Azuma K, et al. Little evidence of transdifferentiation of bone marrow-derived cells into pancreatic beta cells. Diabetologia 2003; 46(10):1366–1374.

49 Mathews V, Hanson PT, Ford E, Fujita J, Polonsky KS, Graubert TA. Recruitment of bone marrow-derived

endothelial cells to sites of pancreatic beta-cell injury. Diabetes 2004; 53(1):91–98.
50 Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418(6893):41–49.
51 Koshizuka S, Okada S, Okawa A, et al. Transplanted hematopoietic stem cells from bone marrow differentiate into neural lineage cells and promote functional recovery after spinal cord injury in mice. J Neuropathol Exp Neurol 2004; 63(1):64–72.
52 Grinnemo KH, Mansson A, Dellgren G, et al. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infracted rat myocardium. J Thorac Cardiovasc Surg 2004; 127(5):1293–1300.
53 Airey JA, Almeida-Porada G, Colletti EJ, et al. Human mesenchymal stem cells form Purkinje fibers in fetal sheep heart. Circulation 2004; 109(11):1401–1407.
54 McBride C, Gaupp D, Phinney DG. Quantifying levels of transplanted murine and human mesenchymal stem cells *in vivo* by real-time PCR. Cytotherapy 2003; 5(1):7–18.
55 Wang JF, Wu YF, Harringtong J, McNiece IK. Ex vivo expansions and transplantations of mouse bone marrow-derived hematopoietic stem/progenitor cells. J Zhejiang Univ Sci 2004; 5(2):157–163.
56 Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? Nat Med 2001; 7:393–395.
57 Lemischka I. A few thoughts about the plasticity of stem cells. Exp Hematol 2002; 30:848–852.
58 Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell 2004; 116:639–648.
59 Quesenberry PJ, Dooner G, Dooner M, Abedi M. Developmental biology: Ignortio Elenchi: red herrings in stem cell research. Science 2005; 308:1121–1122.
60 Chang JC, Summer R, Sun X, Fitsimmona K, Fine A. Evidence that bone marrow cells do not contribute to the alveolar epithelium. Am J Respir Cell Mol Biol 2005; 33:335–342.
61 Kotton DN, Fabian AJ, Mulligan RC. Failure of bone marrow to reconstitute lung epithelium. Am J Respir Cell Mol Biol 2005; 33:328–334.
62 Sampaolesi M, Torrente Y, Innocenzi A, et al. Cell therapy of α-sarcoglycan null dystrophic mice through intra-arterial delivery of mesangioblasts. Science 2003; 301:487–492.
63 Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 2004; 200(2):123–135.
64 Harris RG, Herzog EL, Bruscia EM, et al. Lack of a fusion requirement for development of bone marrow-derived epithelia. Science 2004; 305(5680):90–93.
65 Wang X, Willenbring H, Akkari Y, et al. Cell fusion is the principle source of bone marrow-derived hepatocytes *in vivo*. Nat Med 2000; 6:1229.
66 Weimann JM, Johansson CB, Trejo A, Blau HM. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. Nat Cell Biol 2003; 5:959–966.
67 Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, et al. Fusion of bone marrow-derived cells with Purkinje neurons, cardiomyocytes, and hepatocytes. Nature 2003; 425:968–973.
68 Newsome PN, Johannessen I, Boyle S, et al. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cell fusion. Gastroenterology 2003; 124:1891–1900.
69 Bailey AS, Jiang A, Afentoulis M, et al. Transplanted adult hematopoietic stem cells differentiate into functional endothelial cells. Blood 2004; 103:13–19.
70 Masuya M, Drake CJ, Fleming PA, et al. Hematopoietic origin of glomerular mesangial cells. Blood 2003; 101:2215–2218.
71 Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 2002; 297:2256–2259.
72 Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Response to comment on "little evidence for developmental plasticity of adult hematopoietic stem cells." Science 2002; 297:2256–2259.
73 Jimenez G, Grifita SD, Ford AM, Greaves MF, Enver T. Activation of the beta-globin locus control region precedes commitment to the erythroid lineage. Proc Natl Acad Sci USA 1992; 89:10618–10622.
74 Kontaraki J, Chen HH, Riggs A, Bonifer C. Chromatin fine structure profiles for a developmentally regulated gene: reorganization of the lysozyme locus before transactivator binding and gene expression. Genes Dev 2000; 14:2106–2122.
75 Bonifer C. Long-distance chromatin mechanisms controlling tissue-specific gene locus activation. Gene 1999; 238:277–289.
76 Maes J, O'Neill LP, Cavelier P, Turner BM, Rougeon F, Goodhardt M. Chromatin remodeling at the Ig loci prior to V(D)J recombination. J Immunol 2001; 167:866–874.
77 Harrison DE, Lemer C, Hoppe PC, Carlson GA, Alling D. Large numbers of primitive stem cells are active simultaneously in aggregated embryo chimeric mice. Blood 1987; 69:773–777.
78 Micklem HS, Lennon JE, Ansell JD, Gray RA. Numbers and dispersion of repopulating hematopoietic cell clones in radiation chimeras as functions of injected cell dose. Exp Hematol 1987; 15:251–257.

79 Harrison DE, Astle CM, Lerner C. Number and continuous proliferative pattern of transplanted primitive pattern of transplanted primitive immunohematopoietic stem cells. Proc Natl Acad Sci USA 1988; 85:822–826.

80 Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988; 241:58–62.

81 Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T. *In vivo* and *in vitro* stem cell function of c-kit and Sca-1-positive murine hematopoietic cells. Blood 1992; 80:3044–3050.

82 Cheshier SH, Morrison SJ, Liao X, Weissman IL. *In vivo* proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci USA 1999; 96:3120–3125.

83 Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. J Exp Med 1996; 183:1797–1806.

84 Wolf NS, Kone A, Priestley GV, Bartelmez SH. *In vivo* and *in vitro* characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine123 FACS selection. Exp Hematol 1993; 21:614–622.

85 Nilsson SK, Dooner MS, Quesenberry PJ. Synchronized cell-cycle induction of engrafting long-term repopulating stem cells. Blood 1997; 90:4646–4650.

86 Reddy GP, Tiarks CY, Pang l, Wuu J, Hsieh CC, Quesenberry PJ. Cell cycle analysis and synchronization of pluripotent hematopoietic progenitor stem cells. Blood 1997; 90:2293–2299.

87 Bradford GB, Williams B, Rossi R, Bertoncello I. Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. Exp Hematol 1997; 25:445–453.

88 Pang L, Reddy PV, McAuliffe CI, Colvin GA, Quesenberry PJ. Studies on BrdU labeling of hematopoietic cells: stem cells and cell lines. J Cell Physiol 2003; 197(2):251–260.

89 Habibian HK, Peters SO, Hsieh CC, et al. The fluctuating phenotype of the lymphohematopoietic stem cell with cell cycle transit. J Exp Med 1998; 188:393–398.

90 Lambert JF, Liu M, Colvin GA, et al. Marrow stem cells shift gene expression and engraftment phenotype with cell cycle transit. J Exp Med 2003; 197(11):1563–1572.

91 Cerny J, Dooner MS, McAuliffe CI, et al. Homing of purified murine lymphohematopoietic stem cells: A cytokine-induced defect. J Hematother Stem Cell Res 2002; 11:913–922.

92 Berrios VM, Dooner GJ, Nowakowski G, et al. The molecular basis for the cytokine-induced defect in homing and engraftment of hematopoietic stem cells. Exp Hematol 2001; 29(11):1326–1335.

93 Becker PS, Nilsson SK, Li Z, et al. Adhesion receptor expression by hematopoietic cell lines and murine progenitors: modulation by cytokines and cell cycle status. Exp Hematol 1999; 27:533–541.

94 Colvin GA, Lambert JF, Moore BE, et al. Intrinsic hematopoietic stem cell/progenitor plasticity: inversions. J Cell Physiol 2004; 199:20–31.

95 Frimberger A, McAuliffe CI, Werme KA, et al. The fleet feet of haematopoietic stem cells: rapid motility, interaction and proteopodia. Br J Haematol 2001; 112:644–654.

96 D'Hondt L, McAuliffe C, Damon J, et al. Circadian variations of bone marrow engraftability. J Cell Physiol 2004; 200:63–70.

97 Kirkland MA. A phase space model of hemopoiesis and the concept of stem cell renewal. Exp Hematol 2004; 32:511–519.

98 Roeder I, Loeffler M. A novel dynamic model of hematopoietic stem cell organization based on the concept of within-tissue plasticity. Exp Hematol 2002; 30:853–861.

99 Jang Y-Y, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol 2004; 6:532–539.

100 Ratajczak J, Miekus K, Kucia M, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia 2006; 20:767–769.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

3

CHAPTER 3

Embryonic stem cells and cardiogenesis

Timothy J. Kamp, MD, PhD, *& Gary E. Lyons,* PhD

Introduction

The isolation of human embryonic stem cells (hESCs) has engendered great excitement and interest in the possibility of an unlimited supply of human cells that could be used in the treatment of a wide range of degenerative diseases. Therapeutic applications employing hESCs to treat a variety of different forms of heart disease are under active preclinical investigation. ESCs have potential advantages as well as disadvantages compared to other cell sources being tested for cardiac repair. The purpose of this chapter is to describe the biological properties of ESCs and the ability of these cells to undergo cardiogenesis *in vitro*. In so doing, the chapter will provide the essential background for appreciating the potential opportunities for cardiac cell therapies using hESCs, as well as describe *in vitro* approaches to overcome some of the barriers to the clinical uses of hESCs.

Discovery and definition of embryonic stem cells

In the 1970s, a cell culture system was sought for studies of early mammalian embryonic development. It was known that postimplantation embryos contained pluripotent cells, but attempts to culture these cells initially failed. Cell lines such as F9, which were derived from teratocarcinomas, were used to study embryo development *in vitro*. Research on these embryonal carcinoma (EC) cell lines established many of the methods that would later be used in the isolation of ESC lines from mice.

Mouse embryonic stem cells (mESCs) were first isolated from the inner cell mass cells of blastocyst stage embryos in 1981 (Figure 3.1). These cells are distinct from EC cells, which have undergone transformation and karyotypic changes. ESCs have an unlimited capacity for self-renewal, have telomerase activity, and express the nuclear proteins Oct4 and Nanog, which define ESC identity. mESCs require the leukemia inhibitory factor (LIF)/STAT3 signaling pathway to remain pluripotent *in vitro* and can be grown on feeder layers of mouse embryo fibroblasts or cell lines that express LIF, such as STO (ATCC, American type culture collection). Alternatively, mESCs can be propagated without feeders with the addition of LIF to the media. Importantly, mESCs are pluripotent, as originally demonstrated by injection of these cells into mouse blastocysts to produce chimeric mice with mESCs contributing to all cell lineages *in vivo* except the trophoblast lineage (Figure 3.1). The ability to form germ line chimeras with genetically modified mESCs provided a major advance in biomedical research by enabling genetic engineering in mice. In addition, pluripotency of mESCs was supported by formation of embryoid bodies (EBs) *in vitro* and by formation of teratomas following injection of mESCs into immunocompromised rats. Both EBs and teratomas contain derivatives of all three primary germ layers.

With the isolation of mESCs, there was great enthusiasm and interest in deriving ESCs from other species including humans, but this proved more difficult than was originally anticipated. It was not until the mid-1990s that ESCs were successfully isolated from nonhuman primates: marmoset

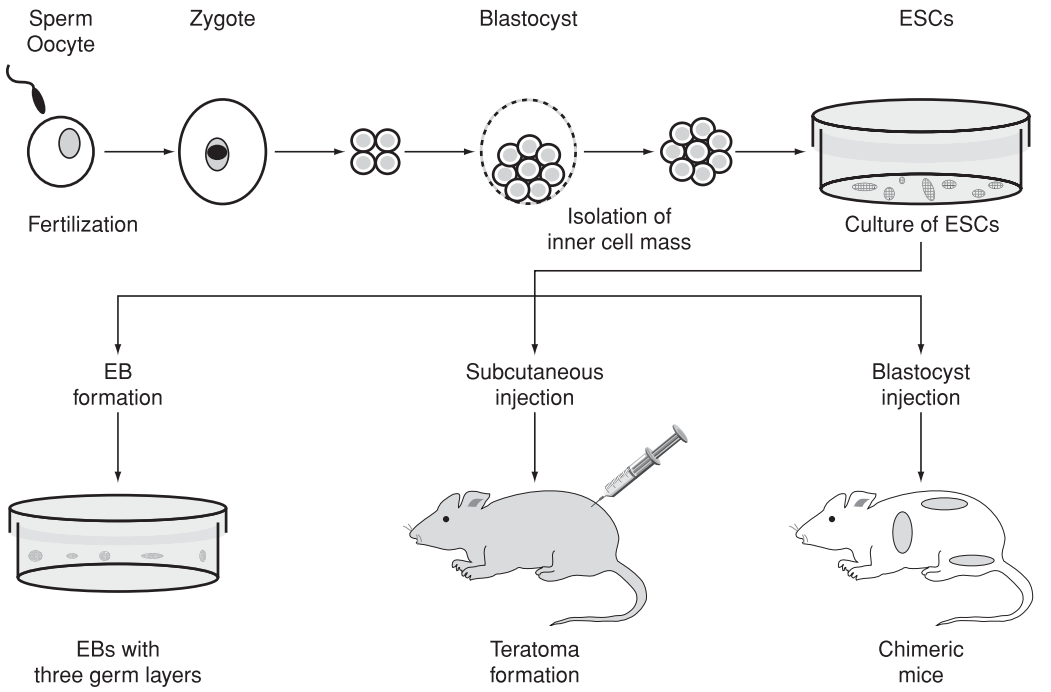


Figure 3.1 Derivation and initial characterization of mouse embryonic stem cells. Fertilization of the oocyte by a sperm produces the zygote, which sequentially divides and forms the blastocyst. The inner cell mass of the blastocyst contains the pluripotent cells (blastomeres) destined to form all tissues of the mouse. The blastomeres can be immunoisolated and cultured under special conditions, producing embryonic stem cells (ESCs). The remarkable pluripotency of ESCs can be demonstrated by *in vitro* embryoid body (EB) formation, teratoma formation, or most rigorously by germ line transmission in chimeric mice.

and rhesus monkeys [1]. On the basis of advances made in the isolation of nonhuman primate ESCs, Thomson and colleagues succeeded in 1998 in isolating hESCs from donated surplus *in vitro* fertilization embryos [2].

Like mESCs, hESCs express Oct4 and Nanog and have telomerase activity. They exhibit self-renewal and proliferate indefinitely while maintaining a normal karyotype. The first hESC lines required a feeder layer of mouse embryo fibroblasts to be maintained in a pluripotent state [2], but now hESCs can be derived and maintained in chemically defined medium without feeder layers [3, 4]. hESCs have been demonstrated to be pluripotent by injection into immunocompromised rats, resulting in the formation of teratomas containing derivatives of all three primary germ layers. *In vitro* differentiation protocols have also confirmed the pluripotent nature of hESCs as described below.

hESCs exhibit several differences from mESCs. hESCs display a number of different cell surface markers and have different growth factor requirements. For example, mouse lines can be maintained in a pluripotent state using LIF. In human lines, LIF is not effective, but basic fibroblast growth factor is required. mESCs double every 8–15 hours, whereas hESCs double every 25–30 hours.

To place ESCs into perspective, it is important to compare them to adult stem cells (ASCs; Table 3.1). ASCs are found in the bone marrow, in the peripheral circulation, or in organs and tissues and differ from ESCs in that they are multipotent but not pluripotent. ASCs are less accessible, proliferate less, undergo senescence related to the age of the donor, and are generally more difficult to propagate in tissue culture. However, for regenerative medicine applications, the potential advantages of ASCs include the possibility of autologous transplantation to avoid immune rejection. The risk of tumorigenicity may be lower for ASCs compared to ESCs. There are minimal ethical/policy concerns with ASC therapy. There are other sources of stem

Table 3.1 Comparison of adult stem cells and embryonic stem cells.

	Embryonic	*Adult*
Source	Human blastocyst/embryo	Adult tissue
Proliferation	Unlimited in theory	Limited
Plasticity	Pluripotent	Multipotent
Tumorigenicity	Concern, teratomas	Less likely
Immunogenicity	Yes for allogeneic	Yes, but autologous
Ethical/public policy	Major debate	Minimal controversy
Efficacy for therapy	Unknown	Unknown
Cost of therapy	Unknown	Unknown

cells that do not neatly fit into the adult and embryonic dichotomy, such as umbilical-cord-blood-derived or testicular stem cells, but these will not be described except to state that their properties are generally intermediate between ASCs and ESCs.

Cardiac development

Because the differentiation of ESCs into cardiomyocytes *in vitro* or after transplantation follows the normal developmental program of cardiogenesis, it is useful to briefly review normal cardiac development. Early in vertebrate development, during the process of gastrulation, the three primary germ layers form in the embryo: endoderm, mesoderm, and ectoderm. Mesoderm gives rise to most of the cells in the mammalian heart. Cardiac progenitors form a structure called the cardiac crescent. Endodermal cells that underlie the cardiac crescent secrete inductive growth factor signals that have a positive role in initiating cardiogenesis. Among these signaling molecules are Notch1, bone morphogenetic protein2, Noggin, transforming growth factor-ß, Wnt11, Nodal, and fibroblast growth factor. In contrast, overlying ectoderm and the neural plate express signaling molecules such as Wnt1 and Wnt3A that inhibit cardiogenesis in adjacent mesoderm. Thus, integrated positive and negative signaling molecules promote cardiogenesis localized specifically in the cardiac crescent [5].

The cardiac crescent then merges at the midline to form the heart as a simple tube that is composed of spontaneously contracting cardiomyocytes lined by endocardium. As the heart undergoes a complex morphogenesis, distinct myocyte types begin to appear: atrial, ventricular, and sinus-nodal cells. Purkinje cells arise as a result of inductive signaling from adjacent endothelial cells. Intercellular signaling from the endocardium and later from the epicardium plays an important role in the differentiation and maturation of the distinct types of cardiomyocytes [6].

mESC cardiac differentiation *in vitro*

In vitro differentiation of mESCs into cardiomyocytes was originally described using an initial aggregation step to form structures referred to as EBs [7]. Differentiated (cystic) EBs include derivatives of all three primary germ layers and can form specialized cell types such as cardiomyocytes and other cell types present in the heart, including endothelial cells and vascular smooth muscle cells [8]. The EB system enables inductive signaling, essential for cardiogenesis to occur; however, there is substantial variability among EBs and only a fraction exhibit cardiogenesis.

Cardiomyocyte differentiation *in vitro* from mESCs occurs in a stepwise fashion, using the EB model (Figure 3.2). There are multiple signaling molecules driving differentiation as those described for embryonic cardiac development, and these result in dramatic changes in cellular phenotype reflected by changes in the expression of hundreds or thousands of genes during the process. Although remarkable progress has been made in identifying many of the critical molecules, we are only at the beginning of unraveling this complex process. Therefore, we will highlight one model of cardiogenesis, indicating some of the most-studied markers for the process, with the acknowledgment that

this model is not uniformly accepted and the details will continue to mature. The process starts with mESCs in EBs beginning to differentiate down the mesodermal pathway, indicated by expression of the transcription factor Brachyury. By day 3 of differentiation, the Brachyury positive cells begin to express the surface tyrosine kinase receptor Flk-1(mouse)/KDR(human), which has been suggested to indicate a population of cardiohemangioblasts, that ultimately can give rise to blood, endothelial cells, smooth muscle, skeletal myocytes, and cardiomyocytes [9, 10]. Some cardiohemangioblasts can commit to blood and endothelial fates as hemangioblasts indicated by the expression of Brachyury along with Scl (a basic helix-loop-helix transcription factor) and Flk-1. The hemangioblast can form blast colonies and can give rise to hematopoiesis as well as endothelial cells. Cardiohemangioblasts can also commit to the cardiac lineage, forming a cardioprogenitor around day 6 and then differentiate further into functional cardiomyocytes, which express the transcription factors Nkx2.5 and GATA-4. Likewise, cardiohemangioblasts can give rise to smooth muscle cells. Therefore, the cardiohemangioblast represents a committed cell type that can give rise to all of the major cell types present in the heart and may represent an appealing cell population to be used in cardiac cellular therapy. Cell surface markers for many of these subpopulations, such as the cardiohemangioblast, cardioprogenitor cells, and cardiomyocytes, are not well defined and are currently a main focus of research on cardiomyocyte differentiation *in vitro*. Identifying cell surface marker proteins will contribute to the long-term ability to identify and purify different populations of cardiac progenitor cells in order to refine myocardial cell therapy applications to use the safest and most effective donor cell populations.

The cardiomyocytes obtained from mESCs have been identified and characterized using gene expression studies, immunohistochemistry for cardiac-specific proteins, ultrastructure analysis, and functional studies evaluating the contractile and electrophysiological properties of the cells [8]. Four distinct types of cardiomyocytes have been

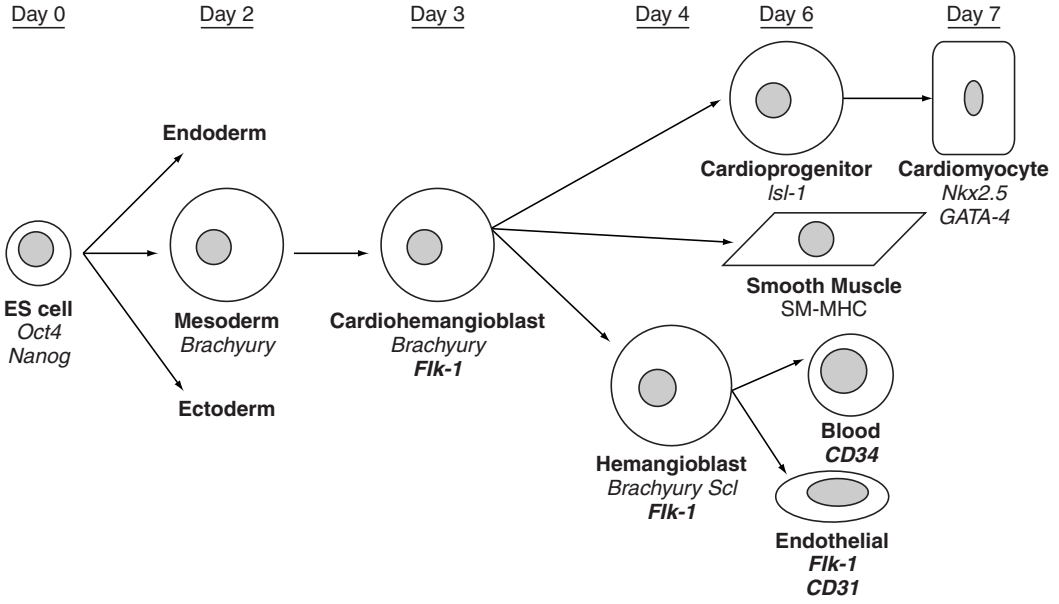


Figure 3.2 Schematic diagram of differentiation of mouse embryonic stem cells (mESCs) in embryoid bodies (EBs) down the mesodermal pathway and relevant cardiovascular cell lineages. The diagram presents a relative time course for differentiation in EBs, with day 0 being the day of formation of the EB. The relevant cell types are indicated with key marker proteins beneath. In italics are transcription factors that help define the cell lineage and in bold italics are cell surface marker proteins.

observed, including nodal-like, ventricular-like, atrial-like, and Purkinje-like myocytes. The cardiomyocytes mature during time in culture from primitive, small, spherical cells with poorly organized myofilament to more elongated flat cells with well-organized bundles of myofilaments. Although the EB system using mESCs has provided a reliable methodology to obtain cardiomyocytes, it is largely inefficient with the cardiomyocytes forming a minority (5–20%) of the cells present and with EBs showing a variety of different cardiomyocyte types.

Inductive coculture and growth factors to enhance cardiogenesis in EBs

Researchers focused on improving the reproducibility and efficiency of obtaining cardiomyocytes from mESCs have explored a number of different approaches. Because inductive signaling is critical for *in vivo* cardiogenesis [11], investigators have studied the impact of coculture of EBs from mESCs with explanted avian precardiac endoderm [12]. This labor-intensive approach resulted in a remarkable increase in the efficiency of obtaining beating EBs. If a combination of chick precardiac endoderm and mesoderm was used for coculture, all of the EBs of the selected size were contractile, with an average of 86% of the cells being cardiomyocytes [12]. This remarkable enhancement in the success of obtaining cardiomyocytes from EBs indicates that there is promise for developing highly efficient protocols; however, the laborious microdissection of chick embryos limits the widespread applicability of this approach. Ideally, the critical signaling molecules released by the avian precardiac endoderm and mesoderm, which are responsible for induction of cardiac differentiation, will be defined and used in future approaches.

Isolation or selection of cardiomyocytes from EBs

Because cardiogenesis in EBs with most protocols has been inefficient, methods to enrich or isolate cardiomyocytes from EBs have been developed. Pioneering work by Field and colleagues described the use of genetically engineered mESCs containing a transgenic cardiac-specific promoter (alpha-myosin heavy chain [α-MHC]) driving the expression of an antibiotic resistance gene (aminoglycoside phosphotransferase) to select cardiomyocytes differentiating *in vitro* from mESCs [13]. By exposing the differentiating EBs to neomycin, this study was able to demonstrate an enrichment of the cells to more than 99% cardiomyocytes. This approach using the α-MHC-neomycin resistance targeted mESC has been extrapolated to protocols generating large numbers of cardiomyocytes in bioreactors ($\sim 10^9$ cardiomyocytes/2 L bioreactor) [14]. But it is critical to appreciate that a range of cellular phenotypes have been identified in the isolated cells, including atrial, nodal, and ventricular myocytes, which was anticipated on the basis of the fact that the promoters used in these studies are active throughout the heart. To obtain ventricular myocytes specifically, two groups have created genetically engineered mESC lines containing reporter genes driven by the rat myosin light chain 2v promoter to obtain ventricular-specific expression of ECFP (enhanced cyan fluorescent protein) or EGFP (enhanced green fluorescent protein) in differentiating mESCs [15, 16]. These studies in mESCs have proven the potential utility of using genetically engineered cell lines with cell-type-specific reporters or antibiotic resistance genes to obtain more homogeneous populations of cardiomyocytes.

Directed cardiac differentiation of mESCs using small molecules

A number of different signaling molecules linked to cardiogenesis have been tested for their ability to enhance cardiac differentiation of mESCs in EBs. Given the critical role of BMP signaling in cardiac development [5], agonists and antagonists for this signaling pathway have been studied. Initially, investigations tested the ability of BMP2 and TGF-β to prime mESCs prior to differentiation in EBs [17]. BMP2 alone and TGF-β alone both resulted in a greater percentage of EBs that were spontaneously beating as well as larger areas of the EBs containing beating cells [17]. However, a more recent study failed to identify a cardiac inductive effect of "priming" mESCs with BMP2 or BMP4, and these investigators instead suggested that a critically timed inhibition of BMP signaling with its antagonist, Noggin, could dramatically enhance cardiac differentiation [18]. Noggin administration to the undifferentiated mESCs at days 3 through day 0 (the day of EB formation) optimally stimulated

cardiogenesis and resulted in 95% of the EBs exhibiting spontaneous contractions and a 100-fold increase in the number of cardiomyocytes [18]. These results highlight the powerful role of BMP signaling in cardiogenesis, but also point to the dynamic nature of this signaling and potentially different role of BMP signaling in mesodermal induction, cardiac commitment, and cardiac differentiation. Studies of other signaling molecules including insulin-like growth factor-1 (IGF-1), FGF, oxytocin, erythropoietin, nitric oxide, and retinoic acid have demonstrated some increase in efficiency of generation of cardiomyocytes from EBs [8], but the effects were relatively meager compared to the recent study using Noggin [18].

An alternative approach to enhancing differentiation of cardiomyocytes from ESCs has used high throughput screening of large chemical libraries of small molecules. The goal is to identify a synthetic small molecule that can act on critical signaling and regulatory molecules to efficiently induce cardiogenesis. These studies have used undifferentiated mESCs that are exposed to the chemical compounds without forming EBs. The first successful screen used a library of 880 compounds approved for clinical use in humans, which were tested on CGR8 mESCs expressing the α-MHC promoter driving EGFP [19]. This study identified a single compound, ascorbic acid (vitamin C), as strongly promoting cardiogenesis. There was a dose-dependent effect, but the exact percentage of cardiomyocytes was not provided. Another small molecule screen of 100,000 heterocyclic molecules identified cardiogenols as powerful compounds that can induce cardiogenesis [20]. In this study more than 50% of the cells stained positive for the myofilament protein MHC, and 90% expressed the cardiac transcription factor GATA-4. Therefore, small molecules may provide a very powerful tool to directly induce cardiac differentiation from mESCs, but the mechanisms involved as well as the reproducibility have not been defined.

In summary, research using mESCs has clearly demonstrated that ESCs can differentiate *in vitro* into functional cardiomyocytes. The most common method of inducing cardiogenesis has been to utilize the formation of EBs, but this process is relatively inefficient and is associated with significant variability. Efforts to optimize the process by testing specific growth factors, cocultures, and small molecules have identified promising leads to make the process efficient. Nevertheless, the results for most of the growth factors and other tested strategies have not been confirmed by multiple laboratories. Many difficult-to-define variables remain, such as the effects of serum components in all of the protocols, differences between the various cell lines used, and the influence of passage number. In the long-term, a combination of growth factors added at specific concentrations in a sequential pattern in defined media are likely to result in the most efficient protocols to generate cardiomyocytes from ESCs.

ESCs and cardiac differentiation

Multiple hESC lines have been shown to differentiate into cardiomyocytes by laboratories around the world [21–23]. However, the efficiency of generating human cardiomyocytes from hESCs and the procedures remain relatively crude compared to the state-of-the-art use of mESCs for cardiogenesis. This reflects the relatively recent isolation of hESCs compared to mESCs and the greater difficulty in culture and maintenance of hESCs as well as the restricted availability of hESCs to investigators around the world because of public policy. In addition, direct extrapolation of the results from mESCs to hESCs may not immediately succeed as initial studies of BMP-2, dimethylsulfoxide, and retinoic acid have not reproduced the apparent successes observed with induction of cardiogenesis in mESCs [21]. Nevertheless, research in this area is rapidly expanding, and significant advances are anticipated in the near future.

Approaches to induce cardiogenesis from hESCs

Induction of cardiogenesis from hESCs has most commonly been performed using EB formation similar to the description for mESCs. The three-dimensional EBs generate all three primary germ layers and provide the critical signaling environment, enabling differentiation of mesodermal cells

into cardiomyocytes. However, there are several differences in the techniques employed for EB formation for hESCs compared to mESCs. hESCs do not survive well if enzymatically isolated to single cells and therefore the hanging drop technique popular with mESC EB formation cannot be readily used. Instead the hESC colonies are lightly digested, frequently with dispase, for aggregates of about 4000 cells, which then are maintained in suspension culture with EB differentiation media, to begin the induction of cardiogenesis. After 4 days, the EBs are plated, and after a total of approximately 8–10 days, spontaneously contracting regions of the EBs are first observed, signifying the presence of differentiating cardiomyocytes. Over the subsequent 10 days, additional contracting areas arise, and the EBs can be maintained in culture for weeks to months as beating EBs.

An alternative method to induce cardiogenesis of hESCs has employed coculture with visceral-endoderm mouse END-2 cells. The secreted signaling molecules provided by the END-2 cells presumably mimic the signaling process that occurs during normal mammalian heart development as the visceral endoderm induces the precardiac mesoderm. Efforts at directed differentiation using defined media without coculture are just beginning [4].

Types and properties of hESC-cardiomyocytes

Unlike many cell types that differentiate from hESCs, cardiomyocytes are readily apparent in culture upon simple inspection, revealing spontaneously contracting cells. The presence of contracting cells provides functional evidence that many of the critical cardiomyocyte-specific proteins are expressed and are functioning appropriately. This has been verified by gene expression studies and by immunolabeling to detect the presence of cardiomyocyte-specific proteins including cardiac troponin-I, atrial natriuretic factor, and others. Electron microscopy has demonstrated varying extents of myofibrillar organization in the developing cardiomyocytes. Characterization of the electrical properties of the cells, using cellular electrophysiology techniques, has provided functional evidence that the hESCs can differentiate into at least three major different types of cardiomyocytes present in the heart—atrial, ventricular, and nodal cells [23, 24]. These conclusions are based on the distinct action potential signature for these different cell types. However, the electrophysiological properties of the hESC-cardiomyocytes (hESC-CMs) characterized from 40 to 90 days of culture are those of embryonic human cardiomyocytes based on more depolarized maximum diastolic potential and slow action potential upstrokes for atrial and ventricular action potentials [23]. Furthermore, the properties of Ca^{2+} cycling in the hESC-CMs studied up to 55 days after differentiation are typical of embryonic mammalian cardiac muscle with influx of extracellular Ca^{2+} driving contraction and little, if any, release of intracellular Ca^{2+} stores with each cardiac cycle [25]. Thus, it is clear that hESCs can differentiate into multiple different types of cardiomyocytes, but currently available techniques have only produced cardiomyocytes exhibiting embryonic phenotypes. The different types of hESC-CMs arise spontaneously in cultures and are not yet able to be isolated as cell-type-specific populations. Ongoing research is focusing on obtaining pure populations of cell-type-specific hESC-CMs at various stages of maturity.

ESC biology and clinical roadblocks

Immunologic rejection

As is the case for solid organ transplantation, immune-mediated rejection represents a major challenge for transplantation of allogeneic cells derived from hESCs. Because somatic cells derived from hESCs are anticipated to express major histocompatibility complex antigens, it is predictable that mismatches between donor and recipient will result in immune-mediated destruction of transplanted cells or tissues. Therefore, it is likely that approaches similar to those used in solid organ transplantation could lead to successful allografting. This would involve human leukocyte antigen (HLA) matching in combination with immunosuppressive therapies. The number of hESC lines needed for such an approach is actively being considered in the strategic planning of stem cell banks. It was recently estimated that 150 consecutive hESC donors would provide a full HLA-A, HLA-B, and

HLA-DR match for a minority (<20%) of recipients, but an HLA-DR match for 85% of recipients, which may be adequate for successful allografts [26]. Thus, there is some feasibility to this approach, but the same problems that plague patients on immunosuppression for solid organ transplants likely would occur in cell transplant recipients, such as increased risks for infection and malignancy. Therefore, alternative strategies to overcome immune rejection for hESC-derived transplants are under active investigation.

The ability to manipulate hESCs in culture prior to transplantation holds great promise for developing new strategies to overcome rejection, which are not readily available for solid organ transplantation. The manipulation in culture could be as simple as developing distinct cells types from the same hESC line that would be used for therapy and induction of tolerance. For example, hESC-CMs could be transplanted into the heart and hESC-derived hematopoietic stem cells or dendritic cells could be transplanted to induce tolerance. This chimerism approach has already shown some promise in animal models of transplantation [27]. Alternatively, the hESCs could be genetically manipulated in a number of ways that may impact their immunogenicity. For example, the cells could be engineered to overexpress immunosuppressive cytokines, or alternatively, the cells could have the genes encoding major histocompatibility antigens knocked out, which theoretically could develop a "universal" donor cell phenotype. However, these genetically engineered hESC lines are hypothetical at this time and will likely require extensive effort to advance.

A final prospect that has garnered great attention is the possibility of somatic cell nuclear transfer technology or therapeutic cloning to develop hESC lines specific for individual patients. Theoretically, the newly derived ESC line and recipient would be genetically and immunologically matched. No immunosuppression would be needed for such an "autologous" transplant. Proof of principle of this approach came in 2001 with the generation of new mESC lines using transfer of somatic cell nuclei [28]. However, the ability to successfully perform somatic cell nuclear transfer with human cells has not yet been clearly demonstrated and represents a continuing challenge. The inefficiency and technical difficulty using this technique to produce individualized hESC lines for each patient makes this approach economically and practically unfeasible at the present time.

Safety

To use hESCs in therapeutic applications requires careful consideration of the safety of this type of therapy. There are at least three major safety considerations that need special attention: risk of infectious complications, risk of tumorigenesis, and tissue-type-specific adverse outcomes such as proarrhythmic effects. To minimize and ideally eliminate these results, a variety of different approaches are under consideration.

Infection

Developing cellular therapies requires that the production of the donor cells strictly follow good manufacturing practices to avoid or minimize the chance of transmission of infectious agents. These guidelines are mandated by the Food and Drug Administration, which must approve cardiac cellular therapies in the United States. In addition to the routine precautions against contamination during cell processing, hESCs bring additional challenges for safety, given the procedures for derivation and maintenance of these cells. There is significant concern regarding the possibility of transmitting pathogens from other animal species to the recipient patients (zoonoses). Potential zoonoses could result from exposure of the hESCs to animal products during derivation and culture. The key examples of animal products include animal serum used in culture and mouse embryonic fibroblasts for feeder layers. Essentially all of the initially derived hESCs lines were exposed to animal products and thus have this potential problem. Although it is possible to remove existing lines from mouse feeder layers and rigorously evaluate them to exclude potential pathogens, it seems likely that newly derived hESC lines that have not been exposed to animal products will be preferable for therapy. These cell lines can be grown on human feeder layers, or as recently demonstrated, without feeder layers using defined media [3, 4]. Once the possibility of zoonoses is eliminated, the procedures for cell processing to avoid infectious agents are well defined and in widespread use for processing a variety of

cells used in therapy such as standard operating protocols for bone marrow processing.

Tumorigenesis

The formation of tumors from transplanted cells is a major potential adverse outcome from cellular therapies. Undifferentiated hESCs are a particular concern because they have been demonstrated to be capable of teratoma formation when transplanted into immunocompromised animals. However, in assessing the potential risk of this outcome, teratomas are generally benign tumors that can be successfully treated in most cases. This is in distinction to highly malignant teratocarcinomas that can arise from transplantation of EC cells. To our knowledge, no cases of teratocarcinomas have been described to result from ESC transplantation. Nevertheless, a variety of strategies to minimize the risk of tumorigenesis are under active investigation, using *in vitro* manipulation of the hESCs. For example, differentiation *in vitro* of hESCs to a committed cardiac progenitor may result in a cell population that is effective for therapy but does not possess the risk for tumorigenesis. Alternatively, genetically engineering hESCs with a gene that will allow targeted killing of tumorigenic cells is under investigation. Such poison pill strategies could provide a safety feature to guard against tumorigenesis. Finally, it is important to realize that any cell source passaged extensively in culture is at risk for acquiring genetic and karyotypic abnormalities, which can transform the cells into a malignant phenotype. Extensive analysis of the hESCs prior to clinical use will need to exclude karyotypic abnormalities or transformation of the cells. However, of the stem cells currently described, ESCs have demonstrated the greatest karyotypic stability. Thus, the risk of tumorigenesis is a significant consideration for therapies with hESCs, but it is also critical to consider this risk with other cell populations that have been maintained in culture prior to transplantation.

Tissue-specific adverse events

Transplantation of cells into a diseased or damaged tissue can sometimes cause tissue-specific adverse effects. The most commonly cited example for heart-directed therapies is the possibility of cell transplantation being proarrhythmic. Conceptually, transplantation of the cells into the heart could contribute to multiple mechanisms of arrhythmia generation, including formation of abnormal sites of pacemaking (abnormal automaticity), abnormalities of conduction leading to reentry, or induction of triggered arrhythmias. Thus, strategies to ensure the proper electrical integration of the transplanted cells as well as ensuring the proper electrophysiological properties of the donor cells are essential. At this stage, our knowledge of this actual risk is limited, and so strategies to combat it have not been significantly developed at the cellular level. However, at the whole organ level in patient trials with some cell types, this risk is addressed by concurrent placement of an implantable cardiodefibrillator to treat the problem if it arises.

Ethics and public policy

A final roadblock to the progress using hESCs for cellular therapies is related to the evolving public policy in many countries. Because of their unique source from a human embryo, hESCs raise important ethical concerns. These concerns are related to the most basic definitions of life and have led to a wide diversity of opinions. However, theologians, ethicists, scientists, and policymakers are moving forward in developing guidelines for the ethical creation and use of hESCs, such as the recent National Academies of Science Guidelines [29]. Nevertheless, there are significant restrictions on research using hESCs, including complete prohibition in some countries, and in the United States, approval for federally funded research using only lines derived before August 9, 2001. The policies are anticipated to continue to evolve, as society gains a better understanding of these cells and as the science continues to advance. For example, the possibility of removing an inner cell mass cell (blastomere) without destroying the viability of the embryo has been successful in deriving new mESC lines [30]. Such an approach may overcome some concerns about embryo loss in derivation of ESCs.

Conclusions and future directions

ESCs are derived from the inner cell mass of *in vitro* fertilized embryos and represent an exciting and potentially powerful cell source for cardiac repair and regeneration. hESCs exhibit the capacity to undergo self-renewal and expansion for potentially

unlimited supplies of human cells, in contrast to adult stem cell sources. Cardiac differentiation of hESCs *in vitro* can result in the formation of multiple distinct types of cardiomyocytes similar to those present in the normal embryonic human heart. Strategies to optimize the efficiency of hESC cardiogenesis are still being developed with multiple potential insights provided by prior studies using mESCs. hESCs can differentiate into cardiac progenitor cells as well as all of the cell types present in the normal heart, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells. Thus, the pluripotent properties of hESCs may enable them or their derivatives to provide more complete cardiac repair or regeneration compared to some other cell types. However, a number of roadblocks remain before clinical applications using hESCs or their derivatives can be safely tested for the treatment of heart disease. Strategies to overcome immune rejection, avoid infectious complications, and minimize the risk of tumorigenesis are under active investigation. As public policy evolves, it is likely that hESCs will increasingly be studied and ultimately tested in clinical applications for myocardial repair.

References

1 Thomson JA, Kalishman J, Golos TG, et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci USA 1995; 92(17):7844–7848.

2 Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282(5391):1145–1147.

3 Ludwig TE, Levenstein ME, Jones JM, et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 2006; 24(2):185–187.

4 Yao S, Chen S, Clark J, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci USA 2006; 103:6907–6912.

5 Zaffran S, Frasch M. Early signals in cardiac development. Circ Res 2002; 91(6):457–469.

6 Lyons GE. Vertebrate heart development. Curr Opin Genet Dev 1996; 6(4):454–460.

7 Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 1985; 87:27–45.

8 Wei H, Juhasz O, Li J, Tarasova YS, Boheler KR. Embryonic stem cells and cardiomyocyte differentiation: phenotypic and molecular analyses. J Cell Mol Med 2005; 9(4):804–817.

9 Iida M, Heike T, Yoshimoto M, Baba S, Doi H, Nakahata T. Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. FASEB J 2005; 19(3):371–378.

10 Motoike T, Markham DW, Rossant J, Sato TN. Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. Genesis 2003; 35(3):153–159.

11 Sugi Y, Lough J. Anterior endoderm is a specific effector of terminal cardiac myocyte differentiation of cells from the embryonic heart forming region. Dev Dyn 1994; 200(2):155–162.

12 Rudy-Reil D, Lough J. Avian precardiac endoderm/mesoderm induces cardiac myocyte differentiation in murine embryonic stem cells. Circ Res 2004; 94(12):e107–e116.

13 Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J Clin Invest 1996; 98:216–224.

14 Schroeder M, Niebruegge S, Werner A, et al. Differentiation and lineage selection of mouse embryonic stem cells in a stirred bench scale bioreactor with automated process control. Biotechnol Bioeng 2005; 92(7):920–933.

15 Muller M, Fleischmann BK, Selbert S, et al. Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB J 2000; 14(15):2540–2548.

16 Meyer N, Jaconi M, Landopoulou A, Fort P, Puceat M. A fluorescent reporter gene as a marker for ventricular specification in ES-derived cardiac cells. FEBS Lett 2000; 478(1–2):151–158.

17 Behfar A, Zingman LV, Hodgson DM, et al. Stem cell differentiation requires a paracrine pathway in the heart. FASEB J 2002; 16(12):1558–1566.

18 Yuasa S, Itabashi Y, Koshimizu U, et al. Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 2005; 23(5):607–611.

19 Takahashi T, Lord B, Schulze PC, et al. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 2003; 107(14):1912–1916.

20 Wu X, Ding S, Ding Q, Gray NS, Schultz PG. Small molecules that induce cardiomyogenesis in embryonic stem cells. J Am Chem Soc 2004; 126(6):1590–1591.

21 Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001; 108(3):407–414.

22 Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from

human embryonic stem cells. Circ Res 2002; 91(6):501–508.

23 He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res 2003; 93(1):32–39.

24 Mummery C, Ward-van Oostwaard D, Doevendans P, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 2003; 107(21):2733–2740.

25 Segev H, Kenyagin-Karsenti D, Fishman B, et al. Molecular analysis of cardiomyocytes derived from human embryonic stem cells. Dev Growth Differ 2005; 47(5):295–306.

26 Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA. Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 2005; 366(9502):2019–2025.

27 Fandrich F, Lin X, Chai GX, et al. Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. Nat Med 2002; 8(2):171–178.

28 Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P. Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 2001; 292(5517):740–743.

29 National Research Council, Institute of Medicine of the National Academies. Guidelines for Human Embryonic Stem Cell Research, 1st edn. Washington, DC: National Academies Press, 2005.

30 Chung Y, Klimanskaya I, Becker S, et al. Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. Nature 2006; 439(7073):216–219.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

4

CHAPTER 4

Mesenchymal stem cell-derived cardiomyogenic cells

Keiichi Fukuda, MD, PhD, *Jun Fujita,* MD, PhD, *Daihiko Hakuno,* MD, PhD, *& Shinji Makino,* MD, PhD

Introduction

Although heart transplantation is currently the ultimate therapy for the treatment of severe heart failure, it is not widely used because of an inadequate supply of donor hearts. As a result, an approach being investigated is the possibility of reversing pump failure by cell transplantation or enhancement of natural regenerative processes. Recent experimental studies have shown that transplanted fetal cardiomyocytes can survive in heart scar tissue and that the transplanted cells have the ability to limit scar expansion and to prevent postinfarction heart failure. As a result of these observations, transplantation of cultured cardiomyocytes into the damaged myocardium has been proposed as a method for treating heart failure [1, 2]. This revolutionary concept remains unfeasible in the clinical setting because of the difficulty in obtaining donor fetal hearts. For this reason, the research focus has shifted to the development of a cardiomyogenic cell line with the capacity of substituting for fetal cardiomyocytes.

Various studies have demonstrated that cardiomyocytes can differentiate from multipotent stem cells (SCs) such as embryonic stem cells (ESCs) [3] and embryonic carcinoma cells [4]. ESCs are an attractive source of progenitor cells for organ regeneration and repair. However, transplanted ESCs are allogeneic, and so recipients must take immunodepressant drugs throughout their lives, which can impact unfavorably on their quality of life. Another disadvantage of ESC transplantation therapy is the potential development of teratocarcinomas from transplanted undifferentiated ESCs. In addition, the establishment of human ESCs poses ethical problems, and research with these cells is banned in certain countries. As a result, studies are being done looking at regeneration of cardiomyocytes from adult autologous SCs.

Recent reports have demonstrated the existence of pluripotent SCs in adult tissues. Roy et al. reported the existence of neural SCs in the brain with the capacity to differentiate into neurons, oligodendrocytes, and astrocytes *in vitro* [5]. Marrow stromal cells possess many characteristics of mesenchymal stem cells (MSCs) [6], and pluripotent progenitor marrow stromal cells can differentiate into various cell types including osteoblasts [7, 8], myocytes [9], adipocytes, tenocytes, and chondroblasts [10]. Our research group recently reported the differentiation of MSCs into cardiomyocytes after exposure of the MSCs to 5-azacytidine, with the subsequent establishment of a cardiomyogenic cell line that can differentiate into cardiomyocytes *in vitro* [11]. Cardiomyogenic cells have been shown to exhibit spontaneous beating and to express both atrial natriuretic peptide and brain natriuretic peptide, and may therefore provide a useful and powerful tool for cardiomyocyte transplantation after further characterization of their cardiomyocyte phenotype.

This chapter describes the characteristics of bone marrow (BM)-derived regenerated cardiomyocytes and discusses the potential applications of these cells in cardiovascular tissue engineering. The expression and function of adrenergic and muscarinic receptors in cardiomyogenic cells is also described,

as these receptors play a critical role in modulating cardiac function [12].

Bone marrow mesenchymal marrow stem cells as a source of cardiomyocytes

BM stromal cells have been used as a feeder layer to culture hematopoietic stem cells (HSCs), and are also known to be of mesodermal origin and produce various cytokines and growth factors. In the late 1990s, a number of investigators reported that BM stromal cells contain multipotent SCs for regenerating nonhematopoietic tissues, called "marrow mesenchymal stem cells," that could differentiate into osteoblasts, chondroblasts, and adipocytes. All these cells were known to be of mesodermal origin. Since MSCs are multipotent, we hypothesized that they might also have the ability to differentiate into cardiomyocytes. As BM cells are obtained directly from the patient, the subsequent use of these cells in the clinical setting would have the advantage that since the cells are autologous, they would not be rejected after cell transplantation.

Method of establishing bone-marrow-derived cardiomyocytes

Mouse BM cells were cultured according to Dexter's method. Following serial passages in culture for more than 4 months, a group of cells escaped senescence. These immortalized cells were subcloned by limiting dilution. Cells were induced to differentiate by treatment with 5-azacytidine. Cells that showed spontaneous beating were subcloned, treated with 5-azacytidine again, and screened a second time for spontaneous beating. Clones with the highest frequency of spontaneous beating were picked, and used to establish the cardiomyogenic cell line.

Phase-contrast micrography and/or immunostaining with antisarcomeric myosin antibodies were used to identify the morphological changes in cardiomyogenic cells. Cardiomyogenic cells showed a fibroblast-like morphology before 5-azacytidine treatment, and this phenotype was retained throughout repeated subculturing under nonstimulating conditions. After 5-azacytidine treatment, the morphology of the cells gradually changed. Approximately 10–30% of the cardiomyogenic cells gradually increased in size after 1 week and developed either a ball-like appearance or lengthened in one direction to exhibit a stick-like morphology. Most of the remaining nonmyocyte cells had an adipocyte-like appearance.

Regenerated cardiomyocytes display a fetal ventricular phenotype

Various cardiac contractile protein isoforms are differentially expressed in cardiomyocytes at different developmental stages and in different chambers. At the time of birth, there is a developmental switch in gene expression of ventricular muscle proteins in small mammals. β-myosin heavy chain (MHC) is the predominant fetal form, while α-MHC is expressed postnatally. There is also a developmental switch from the expression of α-skeletal actin, which is the predominant fetal and neonatal form, to that of α-cardiac actin, the predominant adult form. We investigated the contractile protein isoforms of BM-derived cardiomyogenic cells to investigate their cardiomyocyte phenotype, and the results are summarized in Table 4.1. Fetal, neonatal, and adult ventricle and atrium were used as controls. Expression

Table 4.1 Isoforms of the contractile proteins in differentiated cardiomyogenic cells.

	Atrium		*Ventricle*			
Developmental stage	*Fetus*	*Adult*	*Fetus*	*Neonate*	*Adult*	*CMG*
α-actin	Skeletal	Cardiac	Skeletal > Cardiac	Skeletal	Cardiac	Skeletal > Cardiac
Myosin heavy chain	$\alpha > \beta$	α	$\beta > \alpha$	$\alpha > \beta$	α	$\beta > \alpha$
Myosin light chain	2a	2a	2v	2v	2v	2v

CMG = cardiomyogenic cell.

of both α- and β-MHC was detected in differentiated cardiomyogenic cells by RT-PCR, with β-MHC expression much greater than that of α-MHC. Cardiomyogenic cells expressed both α-*cardiac* and α-*skeletal actin*, but α-*skeletal actin* was expressed at markedly higher levels than α-*cardiac actin*. Also, cardiomyogenic cells expressed myosin light chain (MLC)-2v but not MLC-2a. MLC-2v is expressed specifically in ventricular cells, while MLC-2a is expressed specifically in atrial cells. Skeletal muscle cells do not express either MHC or MLC-2v. These results would indicate that differentiated cardiomyogenic cells are phenotypically similar to fetal ventricular cardiomyocytes [11].

Developmental stage of undifferentiated and differentiated cardiomyogenic cells

Various cardiac-specific transcription factors are serially expressed in the developing heart during myogenesis and morphogenesis. The genes encoding Nkx2.5, GATA4, HAND1/2, and MEF2-B/C are expressed in the early stage of heart development, while MEF2-A and MEF2-D are expressed during the middle stage of heart development. Cardiomyogenic cells express GATA4, TEF-1, Nkx2.5, HAND, and MEF2-C before exposure to 5-azacytidine, and MEF2-A and MEF2-D after exposure to 5-azacytidine. This pattern of gene expression is similar to that of developing cardiomyocytes *in vivo* [11] and indicates that the developmental stage of undifferentiated cardiomyogenic cells is close to that of cardiomyoblasts or the early stages of heart development. The estimated stage of differentiation of cardiomyogenic cells is between the cardiomyocyte-progenitor stage and the differentiated cardiomyocyte stage.

Serial changes in action potential shape in cardiomyogenic cells simulate those of fetal ventricular cardiomyocytes *in vivo*

Cardiomyogenic cells exhibit at least two types of distinguishable morphological action potentials: sinus-node-like potentials and ventricular-myocyte-like potentials [11]. The cardiomyocyte-like action potential recorded from these spontaneous beating cells is characterized by (1) a relatively long action potential duration or plateau (2), a relatively shallow resting membrane potential, and (3) a pacemaker-like late diastolic slow depolarization. Peak-and-dome-like morphology was observed in ventricular-myocyte-like cells. At 3 weeks, only sinus-node-like action potentials were recorded in CMG cells. Ventricular-myocyte-like action potentials were first recorded after 4 weeks, and then gradually increased.

The observation of several distinct patterns of action potential in cardiomyogenic cells may reflect different developmental stages. Yasui et al. studied action potentials and the occurrence of one of the pacemaker currents, I(f), by the whole-cell voltage and current-clamp technique at the stage when a regular heartbeat is first established (9.5 days postcoitum) and at 1 day before birth [13]. They showed a prominent I(f) in mouse embryonic ventricles in the early stage, then a decrease by 82% before birth, concurrent with the loss of regular spontaneous activity by the ventricular cells. They concluded that the I(f) current of the sinus node type is present in early embryonic mouse ventricular cells. Loss of the I(f) current during the second half of embryonic development is associated with a tendency for the ventricle to lose pacemaker potency. Our findings in cardiomyogenic cells may reflect the developmental changes in action potentials that occur in embryonic ventricular cardiomyocytes.

Expression and function of α_1-adrenergic receptors in cardiomyogenic cells

The α- and β-adrenergic receptors play a key role in modulating cardiac hypertrophy and cardiac function, such as heart rate, contractility, and conduction velocity. Cardiomyogenic cells express all the α_1 receptor subtypes (α_{1A}, α_{1B}, and α_{1D}) before 5-azacytidine exposure [12]. The expression pattern of these receptors in undifferentiated cardiomyogenic cells is consistent with their ubiquitous or wide expression *in vivo* [14]. A low level of expression of α_{1A} was observed before 5-azacytidine exposure, and it increased markedly after exposure. Expression of α_{1B} was unaffected by 5-azacytidine. A high level of expression of α_{1D} was detected

Table 4.2 Isoproterenol increased the spontaneous beating rate and contractility of cardiomyogenic cells, mainly via β_1 receptors.

		Isoproterenol (10^{-7} mol/L)			
	Control	*Vehicle*	*Propranolol (10^{-7} mol/L)*	*CGP20712A (10^{-7} mol/L)*	*ICI118551 (10^{-7} mol/L)*
Increase in beating rate (%)	—	47.6 ± 8.4*	10.0 ± 1.9†	13.8 ± 2.4†	37.6 ± 1.9‡
Cell motion (μm)	5.0 ± 0.3	6.8 ± 0.7*	5.6 ± 0.8‡	5.3 ± 0.6‡	ND
Shortening (%)	6.9 ± 0.5	8.5 ± 1.2*	7.2 ± 0.8‡	5.6 ± 0.6‡	ND
Contractile velocity (μm/s)	71.1 ± 5.2	100.9 ± 11.0*	71.3 ± 8.8‡	70.6 ± 6.6‡	ND

Cardiomyogenic cells at 4 weeks after 5-azacytidine exposure were initially exposed to prazosin (10^{-6} mol/L) for 30 minutes to block α_1-adrenergic receptors. Cells were then preincubated for 20 minutes with vehicle (PBS), propranolol, CGP20712A, or ICI118551, and then stimulated with isoproterenol. The beating rate was counted 3 minutes after stimulation. Contractile parameters were analyzed 90 seconds after stimulation. Each contractile parameter value was calculated as the mean of three randomly selected beats in one cell. PBS was added to the control. Values are means ± SE ($n = 100$, each).
* $p < 0.05$ vs. control.
† $p < 0.01$ vs. vehicle (isoproterenol only).
‡ $p < 0.05$ vs. vehicle; ND = not determined.

before 5-azacytidine exposure, but it decreased considerably after exposure. This switch in transcriptional activity may be the result of the cardiomyogenic cells acquiring a cardiomyocyte phenotype. The ventricular cardiomyocytes *in vivo* mainly expressed α_{1A} and α_{1B}, and expressed a low level of α_{1D} receptor. The temporal changes in expression of α_1-adrenergic receptor subtypes in cardiomyogenic cells are very similar to the postnatal changes observed in neonatal rat heart [15, 16].

ERK1/2 was activated by phenylephrine, an α_1 stimulant, within as little as 5 minutes, and the activation peaked at 10 minutes. The phenylephrine-induced phosphorylation was completely inhibited by prazosin. Phenylephrine increased the cell area and perimeter of the cardiomyogenic cardiomyocytes.

Expression and function of β_1- and β_2-adrenergic receptors in cardiomyogenic cells

The cardiomyocytes of the mammalian heart express both β_1- and β_2-adrenergic receptors, the β_1 receptor being the predominant subtype (approximately 75–80% of total β receptors) [17]. Cardiomyogenic cells do not express β_1 and β_2 receptor transcripts prior to 5-azacytidine exposure, but do so after 1 week [12]. Cardiomyogenic cells express β_1 and β_2 mRNA after acquiring the cardiomyocyte phenotype. The temporal pattern of expression of the α_1 receptor differs from that of the β receptors.

Isoproterenol (a β receptor stimulant) increases the cAMP level in cardiomyogenic cells, while propranolol (a nonselective β blocker) completely inhibits isoproterenol-induced cAMP accumulation. Isoproterenol also increases the rate of spontaneous beating (Table 4.2) to a rate 48% higher than control cells [12]. This increase is consistent with that of adult murine cardiomyocytes and ESC-derived cardiomyocytes. Preincubation with propranolol or CGP20712A (a β_1-selective blocker) strongly reduces the isoproterenol-induced increase in the beating rate, while preincubation with ICI118551 (β_2-selective blocker) only slightly decreases the beating rate.

We also investigated the effect of isoproterenol on the contractile function of cardiomyogenic cells and found that it increased cell motion distance, percent shortening, and contractile velocity. The isoproterenol-induced increase in contractility was almost completely inhibited by both propranolol and CGP20712A. Collectively, these results indicate that the β_1- and β_2-adenergic receptors expressed in cardiomyogenic cells are functional, and that the isoproterenol-induced increase in the spontaneous beating rate and in contractility is mainly mediated by β_1 receptors. The β_1 receptor was the

predominant subtype that mediated changes in the beating rate in cardiomyogenic cells, and both the beating rate and the contractility were significantly increased by isoproterenol, and completely inhibited by propranolol and CGP20712A. β_1-receptors play a critical role in mediating the isoproterenol-induced signaling in differentiated cardiomyogenic cells. This expression pattern was consistent with that of cardiomyocytes *in vivo*.

Cardiomyogenic cells express muscarinic receptor mRNA after cardiomyocytes differentiation

Heart rate, conduction velocity, and contractility are negatively regulated by the parasympathetic nervous system in cardiomyocytes, and muscarinic (cholinergic) receptors play an important role in mediating this function. To date, five subtypes of muscarinic receptors (M_1–M_5) have been cloned. The expression of the muscarinic receptors is tissue-specific, and cardiomyocytes express mainly the M_2 receptor subtype in mouse and human [18]. The M_1 receptor subtype is also expressed in murine neonatal and adult cardiomyocytes. Cardiomyogenic cells express these receptors when they acquire the cardiomyocyte phenotype, but express neither prior to 5-azacytidine exposure.

M_1 receptors couple to Gq/G_{11} and activate phospholipase Cβ via G$q\alpha$, leading to IP_3 production, while M_2 receptors couple to $Gi/G_0/Gz$ and activate phospholipase Cβ via G$i\beta\gamma$, leading to IP_3 production [19, 20]. Carbachol, an acetylcholine homologue, increases the content of a second messenger, IP_3, in cardiomyogenic cells, and preincubation with atropine (nonselective muscarinic blocker) and AFDX116 (M_2-selective blocker) inhibit the carbachol-induced IP_3 production. These findings indicate that muscarinic receptors can transduce their signals and that M_2 receptors play a critical role in carbachol-induced IP_3 production in cardiomyogenic cells. This expression pattern is consistent with that of cardiomyocytes *in vivo*.

Cell transplantation therapy for the treatment of heart failure

The concept of cardiomyocyte transplantation has been advocated since the late 1990s [21]. Fetal or neonatal rat cardiomyocytes have been successfully transplanted into the hearts of adults; transplanted cells are viable within the heart for relatively long periods and form gap junctions with surrounding recipient cells. Prior to the transplantation of regenerated cardiomyocytes into the heart, undifferentiated cells and cells that have differentiated into other cell types must be eliminated. Methods that use a cell sorter or drug-resistant genes to collect ESC-derived cardiomyocytes have been reported [22, 23]. These include methods that specifically label cardiomyocytes including the introduction into ESCs of green fluorescent protein (GFP) under the control of a cardiomyocyte-specific promoter. In a previous study, we placed GFP under the control of the MLC gene promoter and introduced it into BM MSCs [24] Figures 4.1A–1S show that when these cells are induced to differentiate, only cardiomyocytes produce the GFP signal and can be collected to a purity of more than 99% with a FACS (fluorescence-activated cell sorter). When these cells are transplanted into the hearts of syngeneic mice, using a syringe, they move into spaces between recipient cardiomyocytes, become connected to surrounding cells via gap junctions, and stably reside in the heart for an extended period of time (Figures 4.1K–1S) [24]. These findings clearly demonstrate the potential of regenerated cardiomyocytes as an alternative to fetal or neonatal cardiomyocytes for transplantation.

In vivo evidence that bone marrow cells generate functional cardiac tissue

In 2001, Orlic et al. demonstrated cardiomyocyte differentiation by transplanting c-kit$^+$Lin$^-$ BM cells into the peri-infarct tissue after myocardial infarction [25]. Further, in 2002, FISH (fluorescence *in situ* hybridization) analysis revealed the presence of numerous recipient-derived cardiomyocytes after a human heart transplantation [26], and in 2003, numerous BM-derived cardiomyocytes were demonstrated in the recipient heart after BM transplantation [27]. In 2004, Balsam and colleagues investigated whether the c-kit$^+$ HSCs in BM were capable of differentiating into cardiomyocytes [28] by directly injecting BM cells into myocardial tissue instead of transplanting BM after irradiation—as other groups had done. Of significance, they

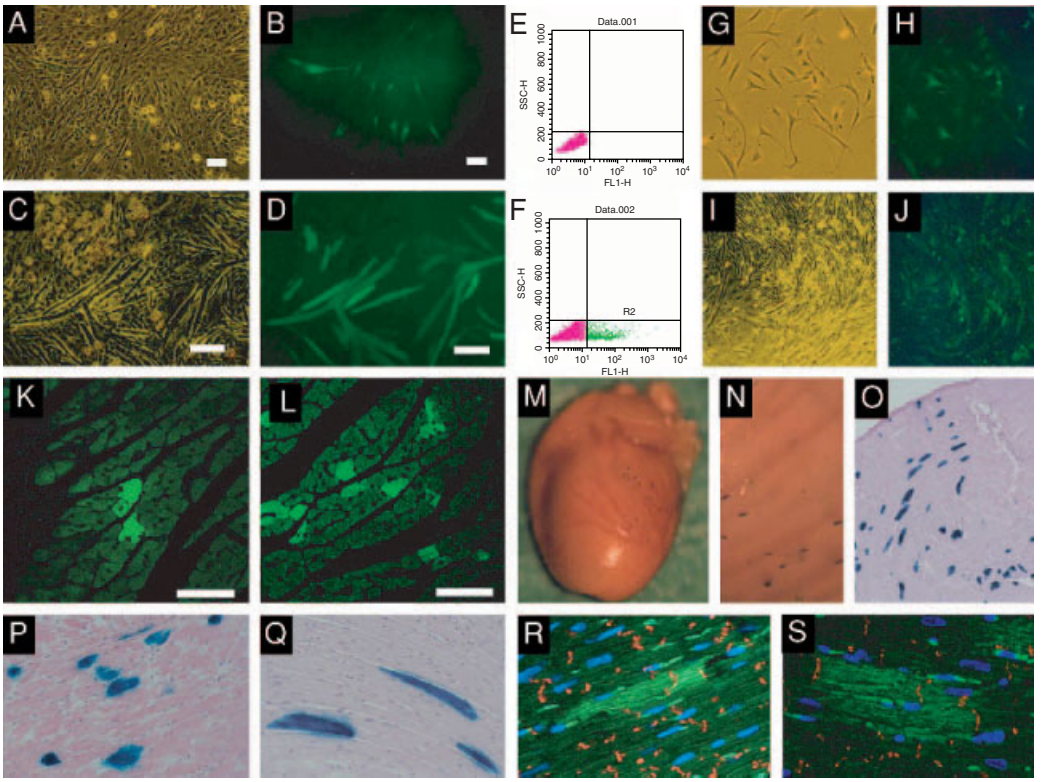


Figure 4.1 Isolation and transplantation of regenerated cardiomyocytes. (A–J) Isolation of regenerated cardiomyocytes. Bone marrow mesenchymal stem cells transfected with plasmid encoding enhanced green fluorescent protein (EGFP) under the control of the promoter of a ventricular-myocardium-specific protein, MLC-2v, and induced to differentiate. Some of the cells are GFP^+ 7 days after differentiation (A and B) and have started beating after 3 weeks (C and D). When the cells were fractionated with a FACS after becoming GFP^+ (E and F), only cardiomyocytes were obtained (G and H). Cardiomyocytes 3 weeks after cell sorting. (I and J) Transplantation of regenerated cardiomyocytes (K–S). Regenerated cardiomyocytes transplanted into adult mice hearts with a syringe. This experiment confirms that transplanted cardiomyocytes stably reside in the heart and survive for a long time. Once injected, the regenerated cardiomyocytes diffuse into the heart-like islands, then closely adhere to surrounding cardiomyocytes and assume the form of mature cardiomyocytes that have the appearance of short strips of paper. Cells displaying GFP fluorescence (K and L). Cells transfected with the *LacZ* gene and stained (M–Q). When these cells were coimmunostained with connexin 43, they formed gap junctions with the surrounding cardiomyocytes (R and S). The green is GFP, the blue is nuclear staining with TOTO-3, and the red represents connexin 43. (Adapted from [24].)

conducted their study in an experimental system that excluded irradiation because of the possibility that invasive treatment, including irradiation, contributed to a fusion phenomenon. They concluded that c-kit^+ HSCs do not include cells capable of differentiating into cardiomyocytes. Murry and colleagues investigated this differentiation ability in a similar manner by directly infusing c-kit^+Lin^- HSCs into the heart [29], and, as expected, showed that HSCs are unable to differentiate into cardiomyocytes.

That same year, we also reported on the differentiation ability of HSCs by using a c-kit^+Sca-$1^+CD34^-Lin^-$ side population ($CD34^-$KSL-SP) of HSCs [30]. When whole BM, which included both HSCs and MSCs, was transplanted from GFP-transgenic mice into lethally irradiated mice and myocardial infarction was induced, very few GFP^+ (BM-derived) cardiomyocytes were found. Interestingly, granulocyte colony-stimulating factor (G-CSF) enhanced the number of GFP^+ cardiomyocytes and nonmyocytes in the infarcted or

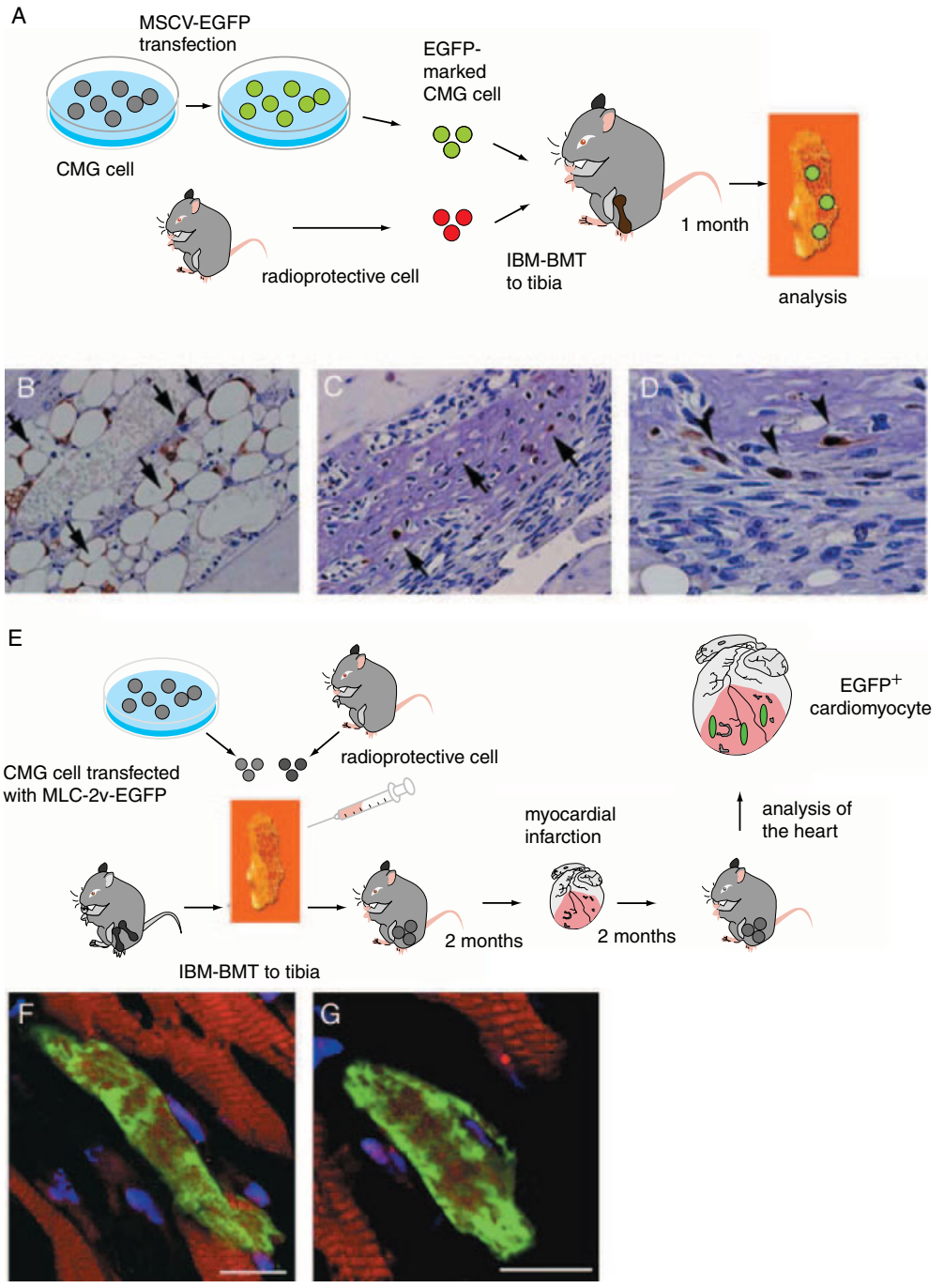


Figure 4.2 Evidence of mobilization and differentiation of BM MSC into cardiomyocytes. GFP-marked MSCs (CMG cell) were transplanted into the intratibial bone marrow space (A). The transplanted CMG cells differentiated into adipocytes (B), osteoblasts (C), and some remained as MSCs (D). EGFP was stained brown with immunohistochemistry. The recombinant plasmid-containing EGFP driven by an MLC-2v promoter was stably transfected into MSCs (E). The cells expressed EGFP upon differentiation into cardiomyocytes. The intra-bone marrow-bone marrow transplantation was performed followed by induction of myocardial infarction and administration of G-CSF. $EGFP^+$ cardiomyocytes were observed in the infarcted and border zone areas (F and G). IBM-BMT = intra-bone marrow–bone marrow transplantation; CMG = cardiomyogenic. (Adapted from [30].)

border zone area. By comparison, when HSC transplantation followed by induction of myocardial infarction and administration of G-CSF were performed, cardiomyocytes were rare in the group transplanted with HSCs alone, although fibroblast-like cells were observed and G-CSF increased their number. Moreover, the presence of predominantly GFP^+ cardiomyocytes derived from MSCs in the group transplanted with cardiomyogenic cells was confirmed, which were purified MSCs. Hence, it was concluded that the differentiation of whole BM into organs (cells) other than hematopoietic populations is attributable to the MSCs and not to the HSCs, and that MSCs are mobilized from the BM into the bloodstream, as are the HSCs (Figure 4.2).

Future direction

The above-mentioned findings provide direct evidence that BM MSCs can regenerate cardiomyocytes. The potential now exists for cardiac tissues damaged by myocardial infarction, or other diseases, to be repaired by BM MSC-derived cardiogenic cells, but the precise mechanism underlying this process needs to be understood to best achieve this goal. Challenges that need to be addressed regarding the clinical application of these cells include the absence of cell surface markers for MSCs and the difficultly experienced in expanding these cells in humans, probably because of difficulties in culture, which may cause early cell senescence. These problems must be overcome to advance the field of cardiovascular regenerative medicine to the next level.

References

1 Soonpaa MH, Koh GY, Klug MG, Field LJ. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1997; 264:98–101.

2 Delcarpio JB, Claycomb WC. Cardiomyocyte transfer into the mammalian heart. Cell-to-cell interactions in vivo and in vitro. Ann N Y Acad Sci 1997; 52:267–285.

3 Wobus AM, Wallukat G, Hescheler J. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation 1991; 48:173–182.

4 Wobus AM, Kleppisch T, Maltsev V, Hescheler J. Cardiomyocyte-like cells differentiated in vitro from embryonic carcinoma cells P19 are characterized by functional expression of adrenoceptors and Ca^{2+} channels. In Vitro Cell Dev Biol Anim 1994; 30:425–434.

5 Roy NS, Wang S, Jiang L, et al. In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med 2000; 6:271–277.

6 Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997; 276:71–74.

7 Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 1994; 161:218–228.

8 Friedenstein AJ, Chailakhyan R, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 1987; 20:263–272.

9 Ferrari G, Angelis GC, Colleta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998; 279:1528–1530.

10 Ashton BA, Allen TD, Howlett CR, et al. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop 1980; 151:294–307.

11 Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999; 103:697–705.

12 Hakuno D, Fukuda K, Makino S, et al. Bone marrow-derived cardiomyocytes (CMG cell) expressed functionally active adrenergic and muscarinic receptors. Circulation 2002; 105:380–386.

13 Yasui K, Liu W, Opthof T, et al. I(f) current and spontaneous activity in mouse embryonic ventricular myocytes. Circ Res 2001; 88:536–542.

14 Alonso-Llamazares A, Zamanillo D, Casanova E, et al. Molecular cloning of alpha 1d-adrenergic receptor and tissue distribution of three alpha 1-adrenergic receptor subtypes in mouse. J Neurochem 1995; 65:2387–2392.

15 Stewart AF, Rokosh DG, Bailey BA, et al. Cloning of the rat alpha 1C-adrenergic receptor from cardiac myocytes. Alpha 1C, alpha 1B, and alpha 1D mRNAs are present in cardiac myocytes but not in cardiac fibroblasts. Circ Res 1994; 75:796–802.

16 Rokosh DG, Stewart AF, Chang KC, et al. Alpha1-adrenergic receptor subtype mRNAs are differentially regulated by alpha1-adrenergic and other hypertrophic stimuli in cardiac myocytes in culture and in vivo. Repression of alpha1B and alpha1D but induction of alpha1C. J Biol Chem 1996; 271:5839–5843.

17 Rockman HA, Koch WJ, Lefkowitz RJ. Cardiac function in genetically engineered mice with altered adrenergic receptor signaling. Am J Physiol 1997; 272:H1553–H1559.

18 Sharma VK, Colecraft HM, Rubin LE, Sheu SS. Does mammalian heart contain only the M2 muscarinic receptor subtype? Life Sci 1997; 60:1023–1029.

19 Nakamura F, Kato M, Kameyama K, et al. Characterization of Gq family G proteins GL1 alpha (G14 alpha), GL2 alpha (G11 alpha), and Gq alpha expressed in the baculovirus-insect cell system. J Biol Chem 1995; 270:6246–6253.

20 Berstein G, Blank JL, Smrcka AV, et al. Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, Gq/11, and phospholipase C-beta 1. J Biol Chem 1992; 267:8081–8088.

21 Li RK, Mickle DA, Weisel RD, et al. Natural history of fetal rat cardiomyocytes transplanted into adult rat myocardial scar tissue. Circulation 1997; 96(suppl II):179–186.

22 Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J Clin Invest 1996; 98:216–224.

23 Muller M, Fleischmann BK, Selbert S, et al. Selection of ventricular-like cardiomyocytes from ES cells *in vitro*. FASEB J 2000; 14:2540–2548.

24 Hattan N, Kawaguchi H, Ando K, et al. Purified cardiomyocytes from bone marrow mesenchymal stem cells produce stable intracardiac grafts in mice. Cardiovasc Res 2005; 65:334–344.

25 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410:701–705.

26 Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. N Engl J Med 2002; 346:5–15.

27 Deb A, Wang S, Skelding KA, et al. Bone marrow-derived cardiomyocytes are present in adult human heart: a study of gender-mismatched bone marrow transplantation patients. Circulation 2003; 107:1247–1249.

28 Balsam LB, Wagers AJ, Christensen JL, et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004; 428:668–673.

29 Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428:664–668.

30 Kawada H, Fujita J, Kinjo K, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 2004; 104:3581–3587.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

5 CHAPTER 5

Bone marrow cells and vascular growth

Takayuki Asahara, MD, PhD

Introduction

Tissue regeneration by somatic stem/progenitor cells has been recognized as a system for the maintenance of homeostasis in many organs. The isolation and investigation of these somatic stem/progenitor cells describes how these cells contribute to postnatal organogenesis. On the basis of their regenerative potency, these somatic stem/progenitor cells are considered a key therapeutic strategy for damaged organs.

Recently, endothelial progenitor cells (EPCs) have been isolated from adult peripheral blood. EPCs share common stem/progenitor cells with hematopoietic stem cells (HSCs), both being derived from bone marrow (BM) and able to incorporate into foci of physiological or pathological neovascularization. The finding that EPCs home to sites of neovascularization and differentiate into endothelial cells (ECs) *in situ* is consistent with *vasculogenesis*—a critical paradigm well described in embryonic neovascularization, but recently proposed in adults—in which a reservoir of stem/progenitor cells contribute to postnatal vascular organogenesis. This discovery has drastically changed our understanding of adult blood vessel formation. Following is an update of EPC biology as well as highlights of their potential utility in therapeutic vascular regeneration.

Postnatal neovascularization

With the discovery of EPCs in the peripheral blood [1, 2], our understanding of postnatal neovascularization has been expanded from angiogenesis to angio/vasculogenesis. As previously described [3], postnatal neovascularization was originally recognized as *angiogenesis*, which is neovessel formation operated by *in situ* proliferation and migration of preexisting ECs. However, the isolation of EPCs resulted in the addition of the new mechanism vasculogenesis, which is *de novo* vessel formation by *in situ* incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs [4] (Figure 5.1). Furthermore, tissue-specific stem/progenitor cells with the ability to differentiate into myocytes or ECs were isolated in skeletal muscle tissue of the murine hind limb, although their origin remains to be elucidated [5]. This finding suggests that the organ of origin of EPCs may not be limited to the BM, and that other tissue-specific stem/progenitor cells may provide *in situ* EPCs.

In the event of minor scale neovessel formation, i.e., slight wounds or burns, *in situ* preexisting ECs causing postnatal angiogenesis may satisfactorily replicate and replace the existing cell population, as ECs exhibit the ability for self-repair that preserves their proliferative activity. Neovascularization through differentiated ECs, however, is limited in terms of cellular life span (Hayflick limit) and their inability to incorporate into remote target sites. In the case of large-scale tissue repair, such as in patients who experience an acute vascular insult secondary to burns, coronary artery bypass grafting, or acute myocardial infarction [6, 7], or in physiological cyclic organogenesis of the endometrium [4], BM-derived or *in situ* EPC kinetics are activated under the influence of appropriate cytokines, hormones, and/or growth factors through the autocrine, paracrine, and/or endocrine systems.

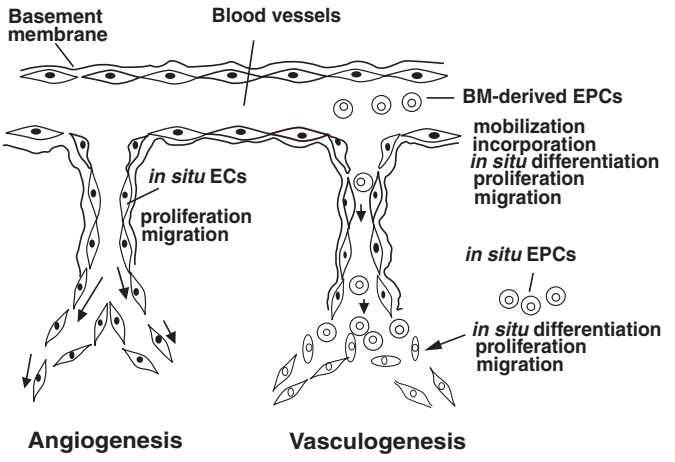


Figure 5.1 Postnatal neovascularization. Postnatal neovascularization in the physiological or pathological event is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of *in situ* ECs and BM-derived or *in situ* EPCs, respectively.

Thus, the contemporary view of tissue regeneration is that neighboring differentiated ECs are relied upon for vascular regeneration during a minor insult, whereas tissue-specific or BM-derived stem/progenitor cells bearing EPCs/ECs are important when an emergent vascular regenerative process is required (Figure 5.1).

Profiles of EPCs in adults

Isolation of EPCs in adults

In the embryo, HSCs and EPCs [8, 9] are derived from a common precursor (hemangioblast) [10, 11]. During embryonic development, multiple blood islands initially fuse to form a yolk sac capillary network [12], which provides the foundation for an arteriovenous vascular system [8]. The integral relationship between the cells which circulate in the vascular system (the blood cells) and those principally responsible for the vessels themselves (ECs) is suggested by their spatial orientation within the blood islands; those cells destined to generate HSCs are situated in the center of the blood island versus EPCs or angioblasts which are located at the periphery of the blood islands. In addition to this arrangement, HSCs and EPCs share certain antigens, including CD34, KDR, Tie-2, CD117, and Sca-1 [13].

The existence of HSCs in the peripheral blood and BM and the demonstration of sustained hematopoietic reconstitution with HSC transplantation led to an idea that a closely related cell type, namely EPCs, may also exist in adult tissues. Recently, EPCs were successfully isolated from circulating mononuclear cells (MNCs) having KDR, CD34, and CD133 antigens shared by both embryonic EPCs and HSCs [1, 14, 15]. *In vitro*, these cells differentiate into endothelial cell lineages, and in animal models of ischemia, EPCs have been shown to incorporate into the foci of neovasculature, contributing to neovascularization. Recently, similar studies with EPCs isolated from human cord blood have demonstrated their ability to differentiate into ECs *in vitro* and *in vivo* [16–19].

These findings have raised important questions regarding the fundamental concepts of blood vessel growth and development in adult subjects. Does the differentiation of EPCs *in situ* (vasculogenesis) play an important role in adult neovascularization, and would impairments in this process lead to clinical disease? There is now a strong body of evidence suggesting that vasculogenesis does in fact make a significant contribution to postnatal neovascularization. Recent studies using animal BM transplantation models in which labeled BM (donor)-derived EPCs could be identified have shown that the contribution of EPCs to neovessel formation may range from 5 to 25% in response to granulation tissue formation [20] or growth factor-induced neovascularization [21]. Also, in tumor neovascularization, EPC participation in vessel formation is approximately 35–45% higher than the former events [22]. The degree of EPC contribution to postnatal neovascularization is variable.

Diverse identifications of human EPCs and their precursors

Since the initial report on EPCs [1, 2], a number of groups have set out to better define this cell population. Because EPCs and HSCs share many surface markers, and no simple definition of EPCs exists, various methods of EPC isolation have been reported [1, 2, 15–18, 23–31]. The term EPC may therefore encompass a group of cells that exist in a variety of stages, ranging from hemangioblasts to fully differentiated ECs. Although the true differentiation lineage of EPCs and their putative precursors are still undetermined, there is overwhelming *in vivo* evidence that a population of EPCs exists in human beings.

Lin and collaborators cultivated peripheral MNCs from patients receiving gender-mismatched BM transplantation and studied their growth *in vitro*. In this study, they identified a population of BM (donor)-derived ECs with high proliferative potential (late outgrowth); these BM cells likely represented EPCs [24]. Gunsilius and coworkers investigated a chronic myelogenous leukemia model and disclosed that BM-derived EPCs contribute to postnatal neovascularization in humans [26]. Interestingly, in the report, BM-derived EPCs could be detected even in the wall of quiescent vessels without neovascularization events. This finding suggests that BM-derived EPCs may also have a role in the normal turnover of ECs, taking place in nondamaged, quiescent blood vessels.

Reyes and collaborators isolated multipotent adult progenitor cells (MAPCs) from BM MNCs, which differentiated into EPCs, and proposed MAPCs as the origin of EPCs [22]. These studies therefore provide evidence to support the presence of BM-derived EPCs that take part in neovascularization. Also, as described above, the existence of *in situ* EPCs derived from tissue-specific stem/progenitor cells in murine skeletal muscle needs to be investigated in other organs [5] (Figure 5.2).

EPC kinetics in adults

EPC kinetics effected by endogenous agents

The incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated through various animal experiments. One well-established model that allows the detection of BM-derived EPCs includes transplanting wild-type mice with BM cells harvested from transgenic mice in which LacZ expression is regulated by an EC lineage-specific promoter, flk-1 or Tie-2 (flk-1/lacZ/BMT, Tie-2/lacZ/BMT). Using such mice, flk-1- or Tie-2-expressing endothelial lineage cells derived from BM (EPCs) have been shown to localize to vessels during tumor growth, wound healing, skeletal and cardiac ischemia, cornea neovascularization, and endometrial remodeling following hormone-induced ovulation [4].

Tissue trauma causes mobilization of hematopoietic cells as well as pluripotent stem/progenitor cells from the hematopoietic system [32]. Consistent with the notion that EPCs and HSCs share a common ancestry, recent data from our laboratory have shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia [8–10]. The aforementioned murine BM transplantation model also provides direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization following the development of hind limb ischemia [4]. This finding indicates that circulating EPCs are mobilized endogenously in response to tissue ischemia and can incorporate into neovascular foci to promote tissue repair. These results in animals were recently confirmed by human studies, illustrating EPC mobilization in patients following burns [6], coronary artery bypass grafting, or acute myocardial infarction [7].

As previous studies have demonstrated the role of endogenous mobilization of BM-derived EPCs, we considered exogenous mobilization of EPCs as an effective means of augmenting the resident population of EPCs/ECs. Such a strategy is appealing for its potential to overcome endothelial dysfunction or depletion that may be present in older, diabetic, or hypercholesterolemic patients. Granulocyte macrophage colony-stimulating factor is well known to activate hematopoietic progenitor cells and myeloid lineage cells, but has recently been shown to exert a potent stimulatory effect on EPC kinetics. The administration of this cytokine was shown to induce EPC mobilization and enhance neovascularization of severely ischemic tissues and *de novo* corneal vascularization [33].

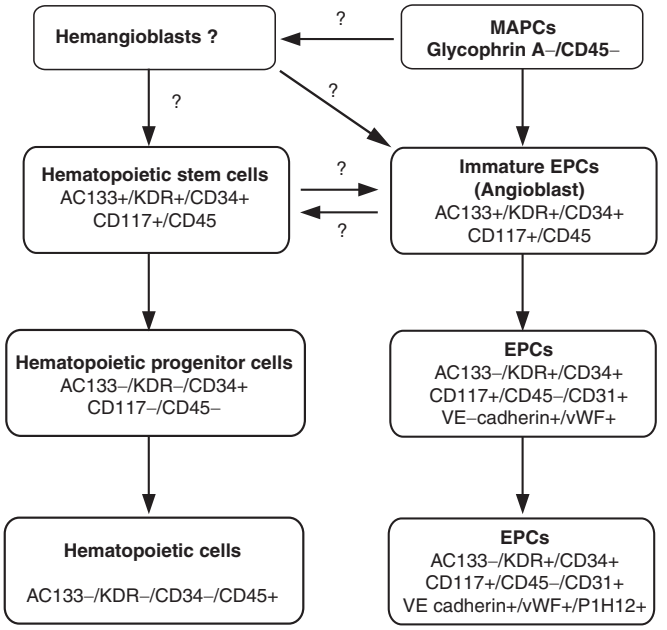


Figure 5.2 Origin and differentiation of EPCs. EPCs are thought to differentiate not only from putative hemangioblasts, common precursor cells with HSCs, as previously described, but also from MAPCs. Representative antigenicities to stem/progenitor cells are shown (+, positive; –, negative).

The exact mechanism by which EPCs are mobilized to the peripheral circulation remains unknown, but may mimic aspects of embryonic development. Vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo [34–36], has recently been shown to be an important stimulus of adult EPC kinetics. Our studies, performed first in mice [37] and subsequently in patients undergoing VEGF gene transfer for critical limb or myocardial ischemia [38], established a previously unappreciated mechanism by which VEGF contributes to neovascularization, that is, in part, dependent on the mobilization of EPCs from the BM. A similar modulation of EPC kinetics has been observed in response to other hematopoietic growth factors, such as granulocyte colony-stimulating factor and stroma-derived factor-1 [39].

EPC kinetics effected by exogenous agents

EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic growth factors but also by pharmacological agents. For instance, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are known to rapidly activate Akt signaling in ECs, thereby stimulating EC bioactivity *in vitro* and enhancing angiogenesis *in vivo* [40]. Recent studies by Dimmeler et al. and our laboratory have demonstrated a novel function of statins, that of mobilizing BM-derived EPCs through the stimulation of the Akt-signaling pathway [41–44]. This newly appreciated pharmacological effect of statins, along with their already well-established effects in treating hypercholesterolemia, suggests a benefit in treating various forms of vascular diseases. On the other hand, as BM-derived EPC kinetics is a critical factor for tumor growth [45], some antiangiogenic agents, e.g., angiostatin or soluble flk-1, inhibit BM-derived EPC kinetics, lead to tumor regression [46].

Clinical profile of EPC kinetics

There is a strong body of evidence to suggest that impaired neovascularization results, in part, from diminished cytokine production. However, endogenous expression of cytokines is not the only factor leading to impaired neovascularization. Diabetic or hypercholesterolemic animals, like clinical patients, exhibit a dysfunction in mature ECs. Although cellular dysfunction does not necessarily preclude a favorable response to cytokine replacement therapy, the extent of recovery in limb perfusion in these

animals fails to reach that of control animals. This latter observation would suggest that a diminished responsiveness of EPCs/ECs to cytokines may limit neovascularization [47–49].

Age-dependent impaired neovascularization [50, 51] might be associated with dysfunctional EPCs and defective vasculogenesis. Indeed, preliminary results from our laboratory indicate that transplantation of BM (including EPCs) from old mice into young mice led to minimal neovascularization in a corneal micropocket assay, with respect to the transplantation of young BM into young recipients. This finding, however, does not exclude the possibility that the age-dependent defect in vasculogenesis may be due to alterations in the tissue microenvironment more than changes in the intrinsic functional properties of EPCs. To answer this important question, we demonstrated that EPCs from older patients with clinical ischemia had significantly less therapeutic effect in rescuing ischemic hind limb of mice compared with those from younger ischemic patients [52]. These studies provide evidence to support an age-dependent impairment in vasculogenesis (as well as angiogenesis) that is heavily influenced by EPC phenotype. Moreover, analyses of clinical data from older patients at our institution revealed a significant reduction in the number of EPCs at baseline, as well as a reduction in response to VEGF165 gene transfer [38]. Thus, impaired EPC mobilization and/or activity in response to VEGF may contribute to the age-dependent defect in postnatal neovascularization. Recently Vasa and collaborators have further investigated EPC kinetics and their relationship to clinical disorders, showing that the number and migratory activity of circulating EPCs inversely correlate with known risk factors for coronary artery disease, such as cigarette smoking, family history, and systemic hypertension [53–55]. On the basis of these findings, monitoring BM-derived EPC kinetics in patients with vascular diseases is expected to be of value in the evaluation of lesion activity and/or therapeutic efficacy.

Therapeutic vasculogenesis

The potential of EPC transplantation

The regenerative potential of stem/progenitor cells is currently undergoing intense investigation. *In vitro*, stem/progenitor cells possess the ability to self-renew and differentiate into organ-specific cell types. When placed *in vivo*, these cells are provided with the proper milieu that allows them to reconstitute organ systems. The novel strategy of EPC transplantation (cell therapy) may therefore supplement the classic paradigm of angiogenesis developed by Folkman and colleagues [3]. Our studies indicate that cell therapy with *in vitro* expanded EPCs can successfully promote neovascularization of ischemic tissues, even when administered as "sole therapy," i.e., in the absence of angiogenic growth factors. Such a "supply-side" version of therapeutic neovascularization in which the substrate (EPCs/ECs) rather than the ligand (growth factor) comprises the therapeutic agent was first demonstrated by intravenously transplanting human EPCs into immunodeficient mice with hind limb ischemia [25]. These findings provided evidence that exogenously administered EPCs could rescue impaired neovascularization in an animal model of critical limb ischemia. Not only did the heterologous cell transplantation improve neovascularization and blood flow recovery, but it also led to important biological outcomes—notably, the reduction of limb necrosis and autoamputation by 50% in comparison with controls. A similar strategy applied to a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs localize to areas of myocardial neovascularization, differentiate into mature ECs, and enhance neovascularization. These findings were associated with preserved left ventricular function and diminished myocardial fibrosis [56]. Murohara et al. also reported similar findings in which human cord-blood-derived EPCs augmented neovascularization in the hind limb ischemic model of nude rats, after *in situ* transplantation [17].

Other researchers have recently explored the therapeutic potential of using freshly isolated human CD34+ cells (EPC-enriched fraction). Schatteman et al. conducted local injections of freshly isolated human CD34+ cells into diabetic nude mice with hind limb ischemia and showed an increase in the restoration of limb flow [29]. Similarly Kocher et al. attempted intravenous infusions of freshly isolated human CD34+ cells into nude rats with myocardial ischemia and found preservation of left ventricular function associated with inhibition of cardiomyocyte apoptosis [57]. Thus, two protocols of

EPC preparation (i.e., cultured and freshly isolated human EPCs) may provide therapeutic benefit in vascular disease, but as described below, will likely require further optimization techniques to acquire the ideal quality and quantity of EPCs for cell therapy.

Recently, Iwasaki et al. have demonstrated CD34+ cell dose-dependent contribution to left ventricular function recovery and neovascularization in the ischemic myocardium [58]. Furthermore, CD34+ cells in higher dose groups committed not only to vasculogenic (endothelial and mural) cell lineages but also to myocyte cell lineages. Clinical trials using mobilized CD34+ may be effective in terms of both vasculogenesis and myocardiogenesis.

Future strategy of EPC cell therapy

Ex vivo expansion of EPCs obtained from peripheral blood MNCs of healthy human volunteers typically yields 5.0×10^6 cells per 100 mL of blood on day 7. Our animal studies [25] suggest that heterologous transplantation requires systemic injection of $0.5 \sim 2.0 \times 10^4$ human EPCs per gram of body weight of the recipient animal to achieve satisfactory reperfusion of an ischemic hind limb. Rough extrapolation of these data to humans suggests that a blood volume of as much as 12 L may be necessary to obtain adequate numbers of EPCs to treat critical limb ischemia in patients. Therefore, the fundamental scarcity of EPCs in the circulation, combined with their possible functional impairment associated with a variety of phenotypes in clinical patients, such as aging, diabetes, and hypercholesterolemia, constitute major limitations of primary EPC transplantation. Considering autologous EPC therapy, certain technical improvements that may help to overcome the primary scarcity of a viable and functional EPC population should include (1) local delivery of EPCs, (2) adjunctive strategies (e.g., growth factor supplements) to promote BM-derived EPC mobilization [33, 37], (3) enrichment procedures, i.e., leukapheresis or BM aspiration, (4) enhancement of EPC function by gene transduction (gene-modified EPC therapy), and (5) culture-expansion of EPCs from self-renewable primitive stem cells of BM or other tissues. Alternatively, if the quality and quantity of autologous EPCs to satisfy the effectiveness of EPC therapy cannot be acquired by the strategies described above, allogeneic EPCs derived from umbilical cord blood or culture-expanded from human embryonic stem cells [17, 59] may be available as sources for supplying EPCs.

Gene-modified EPC therapy

A strategy that may prevent potential EPC dysfunction in ischemic disorders is considered reasonable, given the findings that EPC function and mobilization may be impaired in certain disease states. Potential strategies include genetic modification of EPCs to overexpress angiogenic growth factors, enhancing the signaling activity of the angiogenic response, and rejuvenating the bioactivity and/or extending the life span of EPCs.

We have recently shown for the first time that in an animal model of limb ischemia, gene-modified EPCs can reverse impaired neovascularization [60]. Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 not only improved neovascularization and blood flow recovery but also had beneficial clinical actions, i.e., limb necrosis and autoamputation were reduced by 63.7% in comparison with controls. Notably, the dose of EPCs needed to achieve limb salvage in these *in vivo* experiments was 30 times less than that required in the previous experiments involving unmodified EPCs [25]. Thus, combining EPC cell therapy with gene (i.e., VEGF) therapy may be one option to address the limited number of functional EPCs that can be isolated from the peripheral blood of patients.

BM-MNC transplantation

Nonselected total BM cells or BM-MNCs including the immature EPC population have also been investigated regarding their potential ability to induce neovascularization. Several investigators have reported that autologous BM administration into rabbit [61] or rat [62], using a hind limb ischemic model or a porcine myocardial ischemic model [63, 64], could augment neovascularization in the ischemic tissue, mainly through the production of angiogenic growth factors and less through the differentiation of a portion of the cells into EPCs/ECs *in situ*. Although there are no long-term safety and efficacy data to support the local delivery of such cell populations that are composed mostly

of inflammatory leukocytes, these strategies are already being applied in patients, and preliminary results are expected soon.

Other potential uses of EPCs

EPCs have recently been applied to the field of tissue engineering as a means of improving the biocompatibility of vascular grafts. Artificial grafts first seeded with autologous CD34+ cells from canine BM and then implanted into aortae were found to have increased surface endothelialization and vascularization compared with controls [65]. Similarly, when cultured autologous ovine EPCs were seeded onto carotid interposition grafts, the EPC-seeded grafts achieved physiological motility and remained patent for 130 versus 15 days in nonseeded grafts [66]. Alternatively, the cell sheets of cultured cardiomyocytes may be effective for the improvement of cardiac function in damaged hearts, i.e., ischemic heart disease or cardiomyopathy [67, 68]. Cell sheets consisting of cardiomyocytes with EPCs inducing neovascularization may be attractive, as the blood supply is essential for maintaining the homeostasis of implanted cardiomyocytes in such cell sheets.

Conclusion

As our understanding of adult BM-derived EPCs and postnatal vasculogenesis is enhanced, the clinical applications of EPC use in regeneration medicine is likely to follow. To optimize both the quality and quantity of EPCs to be used for regeneration, several issues remain to be elucidated, such as the development of more efficient methods of EPC purification and expansion and finding the most effective mode for EPC administration. Alternatively, in patients with impaired BM function, as seen with senescence and/or disease, the numbers of EPCs required for therapy need to be optimized either from umbilical cord blood or from those cells obtained by differentiation from tissue-specific stem/progenitors or embryonic stem cells. The unlimited potential for EPCs, along with the emerging concepts of autologous cell therapy with gene modification, suggests that these approaches may one day be a clinical reality for the treatment of patients with cardiovascular disease.

References

1 Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275:964–967.

2 Shi Q, Rafii S, Wu MH, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998; 92:362–367.

3 Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992; 267:10931–10934.

4 Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85:221–228.

5 Tamaki T, Akatsuka A, Ando K, et al. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J Cell Biol 2002; 157:571–577.

6 Gill M, Dias S, Hattori K, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+) AC133(+) endothelial precursor cells. Circ Res 2001; 88:167–174.

7 Shintani S, Murohara T, Ikeda H, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 2001; 103:2776–2779.

8 Risau W, Sariola H, Zerwes HG, et al. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development 1988; 102:471–478.

9 Pardanaud L, Altmann C, Kitos P, et al. Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development 1987; 100:339–349.

10 Flamme I, Risau W. Induction of vasculogenesis and hematopoiesis in vitro. Development 1992; 116:435–439.

11 Weiss MJ, Orkin SH. In vitro differentiation of murine embryonic stem cells. New approaches to old problems. J Clin Invest 1996; 97:591–595.

12 Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995; 11:73–91.

13 Choi K, Kennedy M, Kazarov A, et al. A common precursor for hematopoietic and endothelial cells. Development 1998; 125:725–732.

14 Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997; 90:5002–5012.

15 Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000; 95:952–958.

16 Nieda M, Nicol A, Denning Kendall P, et al. Endothelial cell precursors are normal components of human umbilical cord blood. Br J Haematol 1997; 98:775–777.

17 Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-derived endothelial precursor cells augment

postnatal neovascularization. J Clin Invest 2000; 105: 1527–1536.
18 Kang HJ, Kim SC, Kim YJ, et al. Short-term phytohaemagglutinin-activated mononuclear cells induce endothelial progenitor cells from cord blood CD34+ cells. Br J Haematol 2001; 113:962–969.
19 Crisa L, Cirulli V, Smith KA, et al. Human cord blood progenitors sustain thymic T-cell development and a novel form of angiogenesis. Blood 1999; 94:3928–3940.
20 Crosby JR, Kaminski WE, Schatteman G, et al. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 2000; 87:728–730.
21 Murayama T, Tepper OM, Silver M, et al. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. Exp Hematol 2002; 30:967–972.
22 Reyes M, Dudek A, Jahagirdar B, et al. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002; 109:337–346.
23 Boyer M, Townsend LE, Vogel LM, et al. Isolation of endothelial cells and their progenitor cells from human peripheral blood. J Vasc Surg 2000; 31:181–189.
24 Lin Y, Weisdorf DJ, Solovey A, et al. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000; 105:71–77.
25 Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 2000; 97:3422–3427.
26 Gunsilius E, Duba HC, Petzer AL, et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet 2000; 355:1688–1691.
27 Gehling UM, Ergun S, Schumacher U, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95:3106–3112.
28 Fernandez Pujol B, Lucibello FC, et al. Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 2000; 65:287–300.
29 Schatteman GC, Hanlon HD, Jiao C, et al. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000; 106:571–578.
30 Harraz M, Jiao C, Hanlon HD, et al. CD34− blood-derived human endothelial cell progenitors. Stem Cells 2001; 19:304–312.
31 Quirici N, Soligo D, Caneva L, et al. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 2001; 115:186–194.
32 Grzelak I, Olszewski WL, Zaleska M, et al. Surgical trauma evokes a rise in the frequency of hematopoietic progenitor cells and cytokine levels in blood circulation. Eur Surg Res 1998; 30:198–204.
33 Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999; 5:434–438.
34 Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; 376:62–66.
35 Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380:435–439.
36 Ferrara N, Carver Moore K, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; 380:439–442.
37 Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999; 18:3964–3972.
38 Kalka C, Masuda H, Takahashi T, et al. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 2000; 86:1198–1202.
39 Moore MA, Hattori K, Heissig B, et al. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. Ann N Y Acad Sci 2001; 938:36–45; discussion 45–47.
40 Kureishi Y, Luo Z, Shiojima I, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 2000; 6:1004–1010.
41 Llevadot J, Murasawa S, Kureishi Y, et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. J Clin Invest 2001; 108:399–405.
42 Dimmeler S, Aicher A, Vasa M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 2001; 108:391–397.
43 Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001; 103:2885–2890.
44 Urbich C, Dernbach E, Zeiher AM, et al. Double-edged role of statins in angiogenesis signaling. Circ Res 2002; 90:737–744.
45 Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001; 7:1194–1201.
46 Davidoff AM, Ng CY, Brown P, et al. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. Clin Cancer Res 2001; 7:2870–2879.

47 Rivard A, Silver M, Chen D, et al. Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. Am J Pathol 1999; 154:355–363.

48 Van Belle E, Rivard A, Chen D, et al. Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines. Circulation 1997; 96:2667–2674.

49 Couffinhal T, Silver M, Kearney M, et al. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE–/– mice. Circulation 1999; 99:3188–3198.

50 Rivard A, Berthou Soulie L, Principe N, et al. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 2000; 275:29643–29647.

51 Rivard A, Fabre JE, Silver M, et al. Age-dependent impairment of angiogenesis. Circulation 1999; 99:111–120.

52 Murayama T, Kalka C, SIlver M, et al. Aging impairs therapeutic contribution of human endothelial progenitor cells to postnatal neovascularization [Abstract]. Circulation 2001; 104:II–68.

53 Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001; 89:E1–E7.

54 Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348:593–600.

55 Werner N, Kosiol S, Schiegl T, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005; 353:999–1007.

56 Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103:634–637.

57 Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001; 7:430–436.

58 Iwasaki H, Kawamoto A, Ishikawa M, et al. Dose-dependent contribution of cd34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery post myocardial infarction. Circulation 2006; 113:1311–1325.

59 Levenberg S, Golub JS, Amit M, et al. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 2002; 99:4391–4396.

60 Iwaguro H, Yamaguchi J, Kalka C, et al. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 2002; 105:732–738.

61 Shintani S, Murohara T, Ikeda H, et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001; 103:897–903.

62 Hamano K, Li TS, Kobayashi T, et al. The induction of angiogenesis by the implantation of autologous bone marrow cells: a novel and simple therapeutic method. Surgery 2001; 130:44–54.

63 Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001; 104:1046–1052.

64 Fuchs S, Baffour R, Zhou YF, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 2001; 37:1726–1732.

65 Bhattacharya V, McSweeney PA, Shi Q, et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells. Blood 2000; 95:581–585.

66 Kaushal S, Amiel GE, Guleserian KJ, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001; 7:1035–1040.

67 Shimizu T, Yamato M, Isoi Y, et al. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 2002; 90:e40.

68 Shimizu T, Yamato M, Akutsu T, et al. Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. J Biomed Mater Res 2002; 60:110–117.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

6

CHAPTER 6

Progenitor cells and vascular repair

Kaushik Mandal, MD, *Marjan Jahangiri,* MS, FRCS, *& Qingbo Xu,* MD, PhD

Introduction

Regenerative medicine is an emerging field of research developed from advances in the understanding of stem cell biology and cellular plasticity. The recent identification of adult multipotential progenitor cells capable of contributing to postinjury repair of a variety of tissues, including the vascular endothelium [1], has led scientists to consider blood vessel wall events like atherosclerosis and angiogenesis as potential targets for cell-based therapies.

Before discussing the potential scope of such cell-based therapies in vascular repair, it is crucial to clarify a few definitions. Stem cells are predominantly present during embryogenesis, with few persisting into adulthood. These cells have a self-renewing capacity and the ability to transdifferentiate into multiple cell types under appropriate conditions. Progenitor (transit) cells represent a subpopulation of cells with varying points of differentiation and with less potential for self-renewal. Progenitor cells are also committed to develop only toward a particular lineage, opposed to the pluripotentiality of stem cells. Accordingly, vascular progenitor cells cannot renew themselves indefinitely and, under appropriate conditions, are able to differentiate into mature vascular cells, e.g., endothelial cells.

In this chapter on the role of progenitor cells in vascular repair, we will begin by appraising the pathophysiology of postinjury repair in normal arteries and venous bypass grafts. Following this, we will attempt to synthesize the current evidence implicating progenitors in atherogenesis and neointimal hyperplasia. Finally, taking cues from pathobiology, we will discuss cell-based strategies for countering the problem of restenosis/graft failure and will conclude with a brief discussion on the unanswered questions/future perspectives in this arena.

Origins of vascular progenitor cells

At present, at least two types of vascular progenitor cells have been identified, endothelial progenitor cells (EPCs) and smooth muscle cell (SMC) precursors [2]. They may exist in different tissues and participate in vascular repair or atherosclerosis. The first evidence suggesting the presence of EPCs in adult circulation was provided by Asahara and colleagues (see also Chap. 5), who showed that mononuclear blood cells from healthy human volunteers could acquire an endothelial phenotype *in vitro* and be incorporated into budding capillaries *in vivo* [3]. These cells were characterized by coexpression of CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2)—antigens shared by both embryonic EPCs and hematopoietic stem cells. However, both CD34 and VEGFR-2 are expressed in mature endothelial cells [4], and hence the continuing search for more unique EPC markers. More recently, hematopoietic progenitor cells that express CD34 and fibroblast growth factor receptor-1, or coexpress CD34, CD133, and VEGFR-3, have been shown to behave as EPCs [5,6].

To explore the possibility that vascular progenitor cells for smooth muscle and endothelium exist in adults, a variety of tissues from apoE-deficient mice were extensively examined. Hu et al. provided the first evidence that the adventitia in aortas harbored

large numbers of cells expressing stem cell markers, e.g., sca-1+ (21%), c-kit+ (9%), CD34+ (15%), Flk1 (4%), but not SSEA-1+ embryonic stem cells, indicating that they are adult stem/progenitor cells [7]. Explanted cultures of adventitial tissues using stem cell medium displayed a heterogeneous outgrowth, e.g., the formation of round-shaped cells surrounded by fibroblast-like monolayer cells. Isolated sca-1+ cells were able to differentiate into SMCs in response to platelet-derived growth factor-BB stimulation *in vitro* [7]. In addition, recent evidence has demonstrated the presence of stem cells in the media of the vessel wall [8]. Thus, a large population of vascular progenitor cells existing in the vessel wall can differentiate into SMCs, which can contribute to the lesion formation of vein graft atherosclerosis.

Recently, several groups reported that fat tissue contains progenitor cells that can differentiate into adipocytes, preadipocytes, vascular endothelial cells, and vascular SMCs and also contains cells that have the ability to differentiate into several lineages, such as fat, bone, cartilage, skeletal, smooth, and cardiac muscle, endothelium, hematopoietic cells, hepatocytes, and neuronal cells [9, 10]. Cloning studies have shown that some adipose-derived stem cells have multilineage differentiation potential. Progenitors are also capable of synthesizing multiple growth factors, including VEGF and hepatocyte growth factor [9]. Early, uncontrolled, nonrandomized clinical research, applying fresh adipose-derived cells into a cranial defect or undifferentiated progenitors into fistulas in Crohn's disease, has shown healing and an absence of side effects [10]. Thus, fat-tissue-harbored progenitor cells may have the potential for differentiating into vascular cells (Figure 6.1) [11].

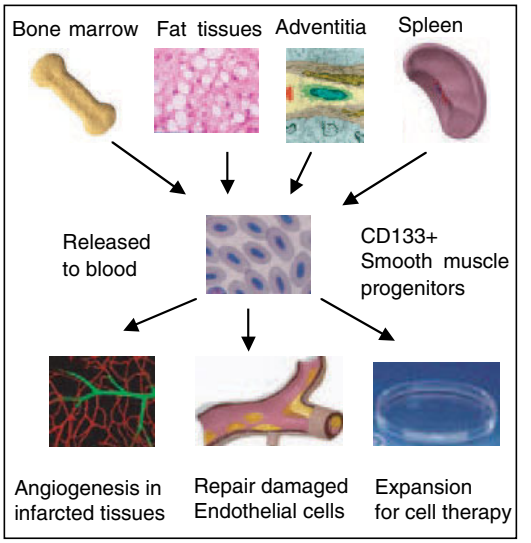


Figure 6.1 Vascular progenitor generation and repair. Vascular progenitors could be released from the bone marrow, fat tissues, the adventitia and possibly from the spleen into the blood, where EPCs express CD133 at the early stage, and then CD34-Flk-1. Circulating progenitors can form neovessels in infarcted tissues, repair damaged endothelial cells of large vessels, and also be expanded *in vitro* for use as cell therapy. (Adapted from [11].)

Vascular injury and repair in normal arteries

Role of the endothelium

The endothelium is a single cell lining covering the internal surface of blood vessels and cardiac valves and plays an important role in thrombogenicity, anticoagulation, leukocyte/platelet adhesion, and vessel contraction and relaxation. It is known that the loss of endothelial cell lining during intervention (harvesting grafts/stent placement) is one of the key events contributing to vascular stenosis. Endothelial cells, when in confluent monolayer, cease to replicate. Disruption of cell contact inhibition results in rapid endothelial cell replication from the proximal and distal nontraumatized segments. There is also some evidence to suggest that progenitor cells may be involved in postinjury endothelial cell replacement [12]. Endothelial cells probably play an important role in controlling smooth muscle proliferation via secretion of heparin and other growth inhibitory factors. Endothelial cells most likely regulate SMC proliferation by the growth inhibitory effects of nitric oxide [13]. Besides modulating local hemostasis and thrombolysis, producing vasoactive compounds, and providing a nonpermeable barrier protecting SMCs against circulating growth promoting factors, endothelial cells themselves synthesize at least three growth factors—fibroblast growth factor , platelet-derived growth factor, transforming growth factor-beta—that are important in smooth muscle proliferation. Endothelial cells also synthesize various components of the basement membrane: type IV and V collagen, laminin,

proteoglycans, and extracellular matrix (fibronectin). Endothelial denudation causes not only endothelial dysfunction, but also exposure of thrombogenic and adhesive subendothelial layers to platelets and leukocytes, with concomitant release of growth factors. These factors may initiate SMC activation, proliferation, and migration to the de-endothelized surface, where they continue to proliferate and secrete extracellular matrix proteins.

With denudation of a small area of endothelial surface, little or no intimal hyperplasia is observed [14]. When larger areas are denuded, however, there is a greater degree of intimal thickening [14]. Haudenschild and Schwartz have demonstrated that SMCs appear in the intima only in areas that are not re-endothelized 7 days after injury [15]. Theoretically, early confluent re-endothelization by EPC seeding may reduce SMC proliferation and/or migration [16].

Role of the media

The arterial media is composed of SMC layer serving as a mechanical structure and giving strength and vasomotor tone to muscular arteries like coronary/carotid arteries. Numerous studies of normal porcine coronary arteries have shown that the neointima does not grow following arterial injury unless the internal elastic lamina is fractured and the arterial media injured [17]. The depth and severity of medial injury generates a proportional neointimal response in both the animal model (pig) and humans. The normal porcine coronary arterial media is typically killed when subjected to temperatures of 80°C for 30 seconds. The resultant medial death is accompanied by voluminous neointimal formation, principally at sites of dead or missing media. Conversely, where arterial media survives the heat, there is little or no neointimal thickening.

The media may have a substantial biological role in limiting neointimal growth. When impermeable, nonporous polymer stents are implanted in normal porcine coronary arteries, they uniformly develop a thick occlusive neointima. In contrast, porous stents fabricated out of identical polymer experienced significantly better patency rates [18]. These data support a regulatory role for the medial SMCs, as only porous design will allow biochemical substances synthesized in media to reach the luminal surface and down-regulate neointimal growth.

Role of the adventitia

The arterial adventitia is the principal connective tissue support to the vessel. It has an important role in providing nourishment and oxygen, via vasa vasorum, to arteries more than 30 cell layers thick. The longitudinal vasa run parallel to the lumen and give rise to secondary branches running circumferentially around the lumen, terminating in the outer medial border. Normally, vasa do not penetrate the media of normal coronary arteries. Atherosclerosis causes adventitial angiogenesis, with small capillaries that frequently penetrate from the adventitia through the media and into atherosclerotic plaque [19]. Normal porcine coronary arteries that are injured develop marked adventitial angiogenesis, proportional to the degree of intimal injury.

The adventitia may play a major role in neointimal formation. Removing the adventitia from a normal artery causes nonobstructive neointimal formation, even in the absence of medial injury [20]. Similarly, placing a tight polymer band around a normal artery also produces neointimal formation. This may result from constriction of adventitial capillaries. However, removing the adventitia and placing a loose polymer collar around a normal artery segment results in formation of a "pseudoadventitia" that limits neointima formation. These studies strongly suggest an important role for the adventitia in metabolically supporting normal arterial function. Adventitial cells not only play a role in the healing of injured arteries, but also seem to be the source of neointimal cells following mechanical injury.

Vascular injury and repair in venous bypass grafts

The earliest cellular event in vein graft atherosclerosis is endothelial cell death [21], probably triggered by the sudden increase in mechanical stress related to systemic arterial pressures. To clarify the source of cells involved in subsequent endothelial regeneration, vein segments from TIE2-*lacZ* transgenic mice expressing the *lacZ* marker gene only in endothelial cells were used for bypass grafting the carotid artery of wild-type mice [22]. These transgenic mice carried *lacZ* gene driven by endothelial TIE2 promoter expressing beta-galactosidase only in endothelial cells and allowed monitoring of the pattern of regeneration of endothelial cells. If, as

originally believed, regeneration of endothelial cells in veins used for bypass grafting were the result of migration from the anastomosed artery, then the earliest sites for *lacZ*-positive cells to appear would be at the ends of the vein segment closest to the artery. However, the analysis of pattern of distribution of these regenerating endothelial cells showed them to be uniformly distributed everywhere on the vein surface. These results suggest that regenerating endothelial cells in venous bypass grafts are likely to originate from recipient circulating blood rather than endothelial cells of recipient/donor vessels [22]. Compared to wild-type mice, apoE knockout mice have a reduced number of circulating progenitor cells, which correlates with poor endothelial regeneration in vein grafts [22].

Corroborative evidence from humans also exists, indicating the role of circulating progenitor cells in replacement of endothelial and SMCs in transplanted vessels [23, 24]. Simper and colleagues described a reduction of EPC colony-forming units, but not a corresponding decrease in circulating endothelial cells in the blood of cardiac allograft patients with established transplant vasculopathy [24]. They further showed that endothelial cells in the atheromas of patients receiving sex-mismatched transplants were of recipient rather than donor origin.

Neointimal hyperplasia—the stem cell hypothesis

Neointimal smooth muscle cells

The appearance of SMCs in the intima is one of the earliest events in the formation of atherosclerotic plaque. It has been observed that SMCs in the atherosclerotic lesion display a more proinflammatory phenotype in contrast to the medial smooth muscles [25]. Does this phenotypic difference between neointimal SMCs and resident medial SMCs imply a different origin? Sata et al. have provided evidence to support this contention and believe that the majority of the neointimal SMCs, as seen in their animal model of atherosclerosis, to be of bone marrow origin [26]. Their conclusion was based on double staining techniques for alpha-actin and markers for stem cells, wherein precise identification of individual cells is difficult. In contrast, our study, which was on SM22-*lacZ* mice expressing the *lacZ* gene only in SMCs, did not support their bone marrow origin [27]. The demonstration of tetraploid SMCs in the blood vessel wall [28] raises the possibility of fusion of homing progenitor cells with intimal SMCs, forming neo-SMCs that have enhanced proliferative and migratory capacity. Despite the controversy, there is support for the notion that circulating progenitor cells do play some role in the development of both the neointima and the resident SMCs [29, 30].

Adventitial cells

Atherosclerosis causes adventitial angiogenesis, and small capillaries arising from the adventitia, penetrating through the media into atherosclerotic plaque are frequently found [19]. In a mouse model of transplant arteriosclerosis, we previously demonstrated that endothelial cells present in microvessels are derived from bone marrow progenitor cells [12]. These data would suggest that apart from their reparative capacity, EPCs may also be the source of cells in the plaque neovasculature, promoting atherosclerosis. Supporting this contention was the recent data from Rotmans and colleagues who demonstrated that *in vivo* seeding of CD34+ progenitor cells on the surface of a polytetrafluoroethylene (PTFE) graft not only promoted endothelization but also stimulated neointimal hyperplasia [31]. Identification of the appropriate EPC and homing mechanisms responsible for recruiting them to the site of endothelial damage/angiogenesis within the vessel wall would enable us to understand their impact and to design therapeutic strategies targeting this process.

Corroborative evidence from human studies

Sufficient data exist to support the role of circulating progenitor cells in causing endothelial and SMC replacement in transplant arteriopathy in humans [23, 24]. Caplice and colleagues, in a postmortem study, examined the coronary arteries of patients who had received sex-mismatched bone marrow transplantation [32]. They could demonstrate sex-mismatched SMCs of donor origin in the coronary plaques, thus supporting the role of progenitor cells in atherosclerosis. Compared to normal controls, the presence of cardiovascular risk factors in patients with symptomatic coronary artery

disease has been shown to correlate with qualitative and quantitative differences in EPCs [33]. It is noteworthy that hypercholesterolemia negatively correlated with EPC numbers and function. Also, Hill and collaborators have reported that EPCs in patients with a higher cardiovascular risk profile are fewer in number, and age faster, compared to those with a lower risk profile [34].

Progenitor-cell-based therapeutic strategies for vascular repair

Does the neointima in a healing artery "know" the lumen size, or whether it is controlled by events in the artery wall? The lumen-based signals that should normally originate from an increasingly tighter stenosis either do not occur or the target tissues are insensitive and fail to stop neointimal thickening before arterial stenosis occurs. Both the critical mass and state of differentiation of neointimal cells may govern their ability to produce appropriate growth-inhibiting signals, and restenosis could be the result of exuberant neointimal proliferation, owing to a lack of appropriate negative feedback. If the above suppositions are correct, then restenosis may be corrected by supplying the appropriate negative signals through "quality" cells, functioning at the site of vascular injury. The artery/vessel must have an active, living lining to prevent neointimal formation or be supplied externally with inhibitory factors.

Endothelial cell transplantation

Technical problems, like the rapid loss of the transplanted cells and difficulty in maintaining cell adherence on restoration of blood flow, hampered initial attempts to re-endothelize denuded areas. In studies of swine femoral arteries, Nabel and colleagues achieved only 2–11% adherence of cells to the denuded arterial wall following 30 minutes of reseeding [35]. Conte et al., using genetically modified venous endothelial cells for reseeding the surface of a balloon-injured rabbit femoral artery, reported significantly enhanced (40–90%) coverage of the denuded intima with transplanted cells [36]. However, their method required surgical exposure of the vessels and complete flow occlusion.

Proceeding on similar lines, various groups have also demonstrated that intravenous infusion of EPCs enhances endothelial healing and reduces neointima formation after vascular injury [37, 38]. Rotmans and colleagues hypothesized that attracting EPCs onto grafts should promote endothelial healing and reported rapid endothelization of CD34-antibody-coated PTFE grafts in a porcine model of arteriovenous fistula [31]. Although an appealing concept, the function of homing EPCs reconstituting the intimal layer is largely unknown, and indeed in the study of Rotmans et al., enhanced endothelization of PTFE grafts with CD34 cells was associated with greater neointima formation [31].

Mobilization of the endogenous resident pool of progenitor cells from bone marrow, using granulocyte colony-stimulating factor, has also been reported to enhance the endothelization and patency of small-caliber prosthetic grafts [39]. However, caution needs to be exercised with this approach as the MAGIC trialists reported an increased rate of in-stent restenosis in coronary angioplasty patients who received granulocyte colony-stimulating factor injections, and the clinical trial had to be terminated prematurely [40].

There is experimental evidence, using statins, for mobilizing the endogenous pool of EPCs to influence the course of atherosclerosis [41]. Despite holding great potential, further exploration into control mechanisms for homing of the right kind of endothelial progenitors and their interaction with paracrine signals and surrounding tissues is necessary and should provide additional insights into this novel approach to treatment of restenosis.

Sources of EPC for therapeutic vasculogenesis

A critical limitation for EPC-based strategies progressing to therapeutic application is their low number in the circulation (see also Chap. 5), which is even lower in patients with cardiovascular risk factors [33, 34]. Approaches to overcome this problem include the use of cord blood or mobilization of EPCs by cytokines, growth factors, or drugs. Umbilical cord blood or granulocyte colony-stimulating factor mobilized blood of adults contains up to tenfold higher number of EPCs [42]. Cord blood cells also have a greater proliferative capacity [42]. VEGF gene transfer has been shown to mobilize EPCs in human subjects [43]. Statins not only enhance the

number of circulating EPCs [41] but also stimulate their incorporation at the site of vascular injury.

Another promising source of progenitor cells for cell-based therapies is adipose tissue [44]. Coating stents with the microvascular endothelial cell fraction of adipose stromal cells has been shown to enhance stent patency in an animal model [45]. The key benefits of using pluripotent adipose stromal cells for autologous cell therapy are the ease with which they can be isolated and their relative abundance. Approximately 10^5–10^6 cells can be isolated from 5 to 10 g of subcutaneous tissue. Because the simple outpatient liposuction can often yield 1 L of fat tissue even in nonobese patients, 10^8–10^9 cells can be isolated from a single individual.

Perivascular endothelial cell therapy

Nugent and Edelman used endothelial cell implants to control vascular repair in a porcine model of arterial injury (balloon injury to carotid) [46]. Seeding endothelial cells at the luminal surface of a blood vessel/graft is technically difficult and allows application of only a limited number of cells at the desired vascular sites. To address these issues, endothelial cells were cultured on three-dimensional polymer matrices (gelfoam), with retention of viability and normal growth kinetics and biochemical activity, and these implants were placed on the adventitial side of the injured blood vessel. Porcine and bovine endothelial implants significantly reduced experimental restenosis by 56 and 31%, respectively, 3 months after angioplasty compared to controls. No implanted cells or focal inflammatory reactions were detected histologically at any of the implant sites at 90 days. A significant increase in antibody titers against endothelium was noted in animals receiving bovine (xenogenic) but not porcine (allogenic) implants. They concluded that endothelial cell implants, even when applied distant from the intimal side, can repair injured endothelium and maintain patency on a long-term basis and that allogenic implants were better than xenogenic implants.

On repeating the same study in a porcine model of arteriovenous fistula [47], these investigators were also able to demonstrate that perivascular allogenic endothelial cell implants, or compounds released by them, reduced neointimal proliferation by influencing early neutrophil infiltration into the anastomotic site. There was no correlation between the effects on intimal hyperplasia and the humoral response in animals with increased levels of circulating antibodies, suggesting that the implants influenced vascular repair in advance of eliciting effective immune responses.

Tissue-engineered neovessels

The ideal small-caliber arterial substitute has to possess specific physiological and mechanical characteristics to support the normal function of the vessel. The discovery of the paracrine release of vasoactive substances such as prostacyclin, prostaglandins, and nitrous oxide, in addition to angiotensin-converting enzyme, anticoagulants, and antifibrinolytics by EPCs, signaled the importance of endothelial cell incorporation into graft design.

Suitability of EPCs for seeding prosthetic grafts to enhance biocompatibility has been suggested in a few recent studies [48]. Griese and colleagues showed that PTFE grafts seeded with EPCs, used for carotid interpositional grafting, were rapidly endothelized [37]. Using a similar strategy, Kaushal et al. showed that the implantation of EPCs onto decellularized porcine iliac vessels, implanted as a coronary interposition graft, reconstituted a functional endothelial layer with improved vasodilatory function and prolonged patency of the grafts [49]. EPCs have also been used in designing bioengineered stents. Shirota and colleagues have reported that EPCs can efficiently seed photo-cured gelatine-coated metallic and microporous thin segmented polyurethane stents [50]. This report raises the potential of using EPC-seeded stents in angioplasty to reduce in-stent restenosis and thrombosis.

Genetically engineered endothelial cell implants

Tissue engineering techniques enable implantation of progenitor cells that have been genetically modified to produce specific compounds. Implants containing genetically modified EPCs [51–53] could then be used to dissect the critical components of vascular response to injury and allow the design of more effective treatment. VEGF 165 gene transfer has been shown to enhance the number of circulating EPCs, thus contributing to vasculogenesis in patients with severe inoperable coronary artery

disease [54]. Kong and colleagues demonstrated that denuded carotid arteries treated with eNOS-expressing EPCs had enhanced inhibition of the neointima and reduced the incidence of thrombosis compared with vessels treated with the control vector [55]. Genetic engineering is also useful in designing strategies to improve EPC homing and survival. Overexpression of human telomerase reverse transcriptase was reported to enhance the proliferative and migratory capacity of EPCs in response to VEGF stimulation, leading to improved neovascularization of the ischemic limb [56].

Perlecan is a major proteoglycan secreted by cultured endothelial cells and is a potent inhibitor of SMC proliferation. Nugent et al. generated a stable clone of bovine aortic endothelial cell, transfected with an antisense vector targeting domain III of perlecan, which produced significantly less perlecan [53]. When Gelfoam matrices containing transfected endothelial cells were implanted adjacent to injured porcine arteries, these cells were less effective at inhibiting neointimal proliferation compared to controls (endothelial cells containing vector only) and also experienced a higher rate of occlusive thrombosis. These results suggest that endothelial control over intimal thickening results from a combination of perlecan and other secreted cell-based products. When one important factor (perlecan) was removed, the implanted cells were completely ineffective at preventing occlusive thrombosis and intimal thickening. These observations may explain why single cell events, thought to be involved in either thrombosis or SMC proliferation, do not lead to full control of vascular homeostasis and fail to translate into meaningful treatments.

Summary and future perspectives

Despite early encouraging results indicating an atheroprotective role for EPCs, several issues need clarification prior to their widespread clinical application. There is an urgent need to standardize protocols for isolation and cultivation of vascular progenitor cells, which in turn mandates that we can identify (specific biomarkers) progenitor cells of interest. To date, much of the experience of progenitor culture has been derived from single cell populations grown in two dimensions. The interactions of multiple cell lines grown in a complex architectural lattice are poorly understood. Clarification is needed regarding the nature of mobilizing, migratory and homing signals, and the mechanisms of differentiation and interaction with the target tissues. Strategies need to be developed to increase the number of vascular progenitors so that an adequate number of cells can be harvested for therapeutic application. We also need to understand how to optimize retention and survival of transplanted cells. The issues of timing of cell administration and the optimal cell numbers needed have to be simultaneously addressed. Large-scale randomized, controlled trials will be essential for defining the clinical conditions most likely to benefit from such therapeutic maneuvers and answer the questions related to the safety of these approaches. The latter issue is of paramount importance, especially given the concerns regarding proatherogenic effects, neovascularization of occult neoplasias, and the potential for aggravating vasculopathies like diabetes. In summary, vascular progenitors have been shown to possess both an antiatherogenic as well as a proatherogenic action. Only a clear understanding of factors controlling these phenomena will allow vascular progenitor-based therapies to ultimately gain wider clinical application.

References

1 Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418:41–49.
2 Xu Q. The impact of progenitor cells in atherosclerosis. Nat Clin Pract Cardiovasc Med 2006; 3:94–101.
3 Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275:964–967.
4 Walter DH, Dimmeler S. Endothelial progenitor cells: regulation and contribution to adult neovascularization. Herz 2002; 27:579–588.
5 Burger PE, Coetzee S, McKeehan WL, et al. Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. Blood 2002; 100:3527–3535.
6 Salven P, Mustjoki S, Alitalo R, et al. VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. Blood 2003; 101:168–172.
7 Hu Y, Zhang Z, Torsney E, et al. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest 2004; 113:1258–1265.

8 Tintut Y, Alfonso Z, Saini T, et al. Multilineage potential of cells from the artery wall. Circulation 2003; 108:2505–2510.

9 Lin Y, Chen X, Yan Z, et al. Multilineage differentiation of adipose-derived stromal cells from GFP transgenic mice. Mol Cell Biochem 2006; 285:69–78.

10 Prunet-Marcassus B, Cousin B, Caton D, Andre M, Penicaud L, Casteilla L. From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res 2006; 312:727–736.

11 Xu QB. Endothelial progenitor cells in angiogenesis. Sheng Li Xue Bao 2005; 57:1–6.

12 Hu Y, Davison F, Zhang Z, Xu Q. Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. Circulation 2003; 108:3122–3127.

13 Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989; 83:1774–1777.

14 Doornekamp F, Borst C, Post MJ. Endothelial cell recovery and intimal hyperplasia after endothelium removal with or without smooth muscle cell necrosis in the rabbit carotid artery. J Vasc Res 1966; 33:146–155.

15 Haudenschild CC, Schwartz SM. Endothelial regeneration. II. Restitution of endothelial continuity. Lab Invest 1979; 41:407–418.

16 Clowes A, Kohler TR. Anatomy, physiology and pharmacology of the vascular wall. In Martin WS (ed.): Vascular Surgery. Philadelphia: WB Saunders, 205–223, 1993.

17 Schwartz RS. Neointima and arterial injury: dogs, rats, pigs, and more. Lab Invest 1994; 71:789–791.

18 Schwartz RS, van der Giessen WJ, Holmes DR, Jr. Biomimicry, vascular restenosis and coronary stents. Semin Interv Cardiol 1998; 3:151–156.

19 Moulton KS. Plaque angiogenesis and atherosclerosis. Curr Atheroscler Rep 2001; 3:225–233.

20 Barker SG, Tilling LC, Miller GC, et al. The adventitia and atherogenesis: removal initiates intimal proliferation in the rabbit which regresses on generation of a "neoadventitia." Atherosclerosis 1994; 105:131–144.

21 Mayr M, Li C, Zou Y, et al. Biomechanical stress-induced apoptosis in vein grafts involves p38 mitogen-activated protein kinases. FASEB J 2000; 14:261–270.

22 Xu Q, Zhang Z, Davison F, Hu Y. Circulating progenitor cells regenerate endothelium of vein graft atherosclerosis, which is diminished in ApoE-deficient mice. Circ Res 2003; 93:e76–e86.

23 Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. N Engl J Med 2002; 346:5–15.

24 Simper D, Wang S, Deb A, et al. Endothelial progenitor cells are decreased in blood of cardiac allograft patients with vasculopathy and endothelial cells of noncardiac origin are enriched in transplant atherosclerosis. Circulation 2003; 108:143–149.

25 Zeiffer U, Schober A, Lietz M, et al. Neointimal smooth muscle cells display a proinflammatory phenotype resulting in increased leukocyte recruitment mediated by P-selectin and chemokines. Circ Res 2004; 94:776–784.

26 Sata M, Saiura A, Kunisato A, et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 2002; 8:403–409.

27 Hu Y, Mayr M, Metzler B, et al. Both donor and recipient origins of smooth muscle cells in vein graft atherosclerotic lesions. Circ Res 2002; 91:e13–e20.

28 Owens GK. Growth response of tetraploid smooth muscle cells to balloon embolectomy-induced vascular injury in the spontaneously hypertensive rat. Am Rev Respir Dis 1989; 140:1467–1470.

29 Shimizu K, Sugiyama S, Aikawa M, et al. Host bone-marrow cells are a source of donor intimal smooth-muscle-like cells in murine aortic transplant arteriopathy. Nat Med 2001; 7:738–741.

30 Xu Y, Arai H, Zhuge X, et al. Role of bone marrow-derived progenitor cells in cuff-induced vascular injury in mice. Arterioscler Thromb Vasc Biol 2004; 24:477–482.

31 Rotmans JI, Heyligers JM, Verhagen HJ, et al. In vivo cell seeding with anti-CD34 antibodies successfully accelerates endothelialization but stimulates intimal hyperplasia in porcine arteriovenous expanded polytetrafluoroethylene grafts. Circulation 2005; 112:12–18.

32 Caplice NM, Bunch TJ, Stalboerger PG, et al. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci USA 2003; 100:4754–4759.

33 Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001; 89:E1–E7.

34 Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348:593–600.

35 Nabel EG, Plautz G, Boyce FM, et al. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 1989; 244:1342–1344.

36 Conte MS, VanMeter GA, Akst LM, et al. Endothelial cell seeding influences lesion development following arterial injury in the cholesterol-fed rabbit. Cardiovasc Res 2002; 53:502–511.

37 Griese DP, Ehsan A, Melo LG, et al. Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. Circulation 2003; 108:2710–2715.

38 Werner N, Junk S, Laufs U, et al. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 2003; 93:e17–e24.

39 Shi Q, Bhattacharya V, Hong-De Wu M, Sauvage LR. Utilizing granulocyte colony-stimulating factor to enhance vascular graft endothelialization from circulating blood cells. Ann Vasc Surg 2002; 16:314–320.

40 Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004; 363:751–756.

41 Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001; 103:2885–2890.

42 Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000; 105:1527–1536.

43 Kalka C, Masuda H, Takahashi T, et al. Vascular endothelial growth factor (165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 2000; 86:1198–1202.

44 Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001; 7:211–228.

45 Williams SK, Rose DG, Jarrell BE. Microvascular endothelial cell sodding of ePTFE vascular grafts: improved patency and stability of the cellular lining. J Biomed Mater Res 1994; 28:203–212.

46 Nugent HM, Edelman ER. Endothelial implants provide long-term control of vascular repair in a porcine model of arterial injury. J Surg Res 2001; 99:228–234.

47 Nugent HM, Groothuis A, Seifert P, et al. Perivascular endothelial implants inhibit intimal hyperplasia in a model of arteriovenous fistulae: a safety and efficacy study in the pig. J Vasc Res 2002; 39:524–533.

48 Shirota T, He H, Yasui H, Matsuda T. Human endothelial progenitor cell-seeded hybrid graft: proliferative and antithrombogenic potentials in vitro and fabrication processing. Tissue Eng 2003; 9:127–136.

49 Kaushal S, Amiel GE, Guleserian KJ, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001; 7:1035–1040.

50 Shirota T, Yasui H, Shimokawa H, Matsuda T. Fabrication of endothelial progenitor cell (EPC)-seeded intravascular stent devices and in vitro endothelialization on hybrid vascular tissue. Biomaterials 2003; 24:2295–2302.

51 Choi JH, Hur J, Yoon CH, et al. Augmentation of therapeutic angiogenesis using genetically modified human endothelial progenitor cells with altered glycogen synthase kinase-3beta activity. J Biol Chem 2004; 279:49430–49438.

52 Cho HJ, Youn SW, Cheon SI, et al. Regulation of endothelial cell and endothelial progenitor cell survival and vasculogenesis by integrin-linked kinase. Arterioscler Thromb Vasc Biol 2005; 25:1154–1160.

53 Nugent MA, Nugent HM, Iozzo RV, et al. Perlecan is required to inhibit thrombosis after deep vascular injury and contributes to endothelial cell-mediated inhibition of intimal hyperplasia. Proc Natl Acad Sci USA 2000; 97:6722–6727.

54 Kalka C, Tehrani H, Laudenberg B, et al. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg 2000; 70:829–834.

55 Kong D, Melo LG, Mangi AA, et al. Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells. Circulation 2004; 109:1769–1775.

56 Murasawa S, Llevadot J, Silver M, et al. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. Circulation 2002; 106:1133–1139.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

PART II

Cardiac progenitor cells in the adult heart

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

7

CHAPTER 7

Cardiac side population cells: phenotype and functional significance

Ronglih Liao, PhD, *Frederic Mouquet,* MD, PhD, & *Otmar Pfister,* MD

Introduction

The characterization of a cell's "stemness" has been based largely upon functional properties including the ability for self-renewal, proliferation, and differentiation into multiple cell types [1]. These unique biological properties are not linked to a single specific molecular marker, and as such, combinations of different stem cell associated markers have been used to identify putative stem cells within a tissue, including the cell surface molecules c-kit, Sca-1, and CD34 [2]. Recently, additional purification strategies, which take advantage of the distinct functional properties of stem cells, have been introduced. Such techniques involve staining with vital dyes such as Rhodamine 123 or Hoechst 33342 [3]. Hoechst staining is commonly used to determine cellular DNA content, as it stoichometrically binds to DNA. The usefulness of Hoechst dye to serve as a stem cell probe relies on the characteristic ability of stem cells to efficiently efflux the dye via ATP-binding cassette (ABC) transporter proteins such as multidrug resistance 1 (Mdr1a/1b in mouse; Mdr1 in human) and ABC superfamily G, member 2 (Abcg2) (also known as breast cancer resistance protein 1, Brcp1) [4–6]. Putative stem cells within Hoechst stained cell suspensions can, therefore, be identified as the cellular fraction with the lowest Hoechst fluorescence intensity upon fluorescence-activated cell sorting (FACS) analysis. Hoechst-low cells characteristically appear to the side of Hoechst dye-retaining cells in the FACS profile, thus and are termed side population (SP) cells [7]. Hoechst efflux by ABC transporters is energy and calcium dependent, and inactivation of the ATP-binding site by fumitremorgin C or calcium inhibition by verapamil efficiently blocks the Hoechst efflux phenomenon [8]. Thus, fumitremorgin C and verapamil are routinely used to document the specificity of the SP profile during FACS analysis.

SP cells were first identified and characterized in murine bone marrow cell suspensions, where they account for 0.05–0.1% of total mononuclear cells [7]. Phenotypic analysis of these cells demonstrated high enrichment for hematopoietic stem cell surface markers, as well as a high degree of stem cell activity, as demonstrated by a 1000-fold increase in *in vivo* reconstitution activity in competitive repopulation experiments [7]. This hematopoietic reconstitution activity is comparable to the enrichment achieved by purification of hematopoietic stem cells using conventional combination of cell surface markers. Therefore, the Hoechst efflux phenomenon has become a highly useful primary purification technique for the isolation of putative hematopoietic stem cells in the absence of cell surface markers.

Over the last decade, tissue resident stem cell populations have become a major focus in stem cell biology. In contrast to the fairly well characterized phenotype of bone-marrow-derived stem cells, stem cell populations in solid organ systems still suffer from the lack of specific cell-surface markers.

Recently, the approach to isolate putative stem cells according to their capacity to efflux Hoechst dye has been successfully translated to various solid tissues. To date, SP cells with documented stem/progenitor cell function have been identified and characterized in various solid organs, including the pancreas, pituitary, testis, mammary gland, lung, liver, skeletal muscle, and recently, the heart (for review see [9, 10]), the latter being the subject of this chapter.

Phenotypic characterization and origin of cardiac SP cells

Cardiac cells exhibiting a verapamil-sensitive SP phenotype were first identified by Hierlihy et al. [11]. Through a slight adaptation of previous bone marrow SP cell isolation techniques, these investigators revealed a Hoechst effluxing subpopulation of cells during FACS analysis of cardiac cell suspensions, similar to that shown in Figure 7.1. Although various groups have since shown the ability to isolate such cardiac SP cells, significant differences in regard to the reported SP cell yield, ranging several orders of magnitude—from 0.03 to 3.5% of total mononucleated cardiac cells—have been reported in the literature [11–15]. Such differences potentially relate to the numerous variables involved in the technical process of SP isolation. Slight variations in tissue digestion, cell counting, Hoechst concentration, and most importantly, the stringency of SP gating may significantly influence the yield of sorted SP cells. In addition to overall yield, significant variability also exists in reported cell surface marker expression for cardiac SP cells [11–15]. We have found a large degree of immunophenotypic variation among cardiac SP cells, particularly in regard to the expression of CD31 (PECAM-1) and to a lesser extent to Sca-1 [14]. Interestingly, immunophenotyic composition as well as the abundance of cardiac SP cells significantly changes with age [11, 15]. Therefore, age-related differences, in addition to differences in SP purification protocols, may account for such variations seen with cardiac SP cell characterization. Consistently, however, adult cardiac SP cells have been shown to express Sca-1 but lack hematopoietic markers such as CD45 and CD34, thus suggesting a nonhematopoietic phenotype [12–14]. Consistent with this phenotype, cardiac SP cells failed to exhibit significant hematopoietic potential when cultured in a medium supporting the formation of hematopoietic colonies [9]. This is in contrast to the SP cell populations found in other solid tissues such as skeletal muscle, liver, and lung, which express CD45 and exhibit concomitant hematopoietic potential [9, 16, 17]. The presence of CD45+ SP cells with hematopoietic potential in these peripheral organs led to the natural hypothesis that solid-tissue-derived SP cells may originate from bone marrow SP cells. Indeed, transplantation experiments with green fluorescent protein (GFP)-expressing bone marrow cells following lethal irradiation in mice have demonstrated a large contribution of bone-marrow-derived (GFP+) SP cells to lung SP cell populations under normal conditions [18]. In contrast, however, bone marrow cells did not contribute significantly to cardiac SP cell populations during normal postnatal heart development [19]. A recent study, using double transgenic mice encoding protein 0-Cre/Floxed-GFP to track neural-crest-derived cells, has suggested that the cardiac neural crest may, in fact, be the origin of neonatal cardiac

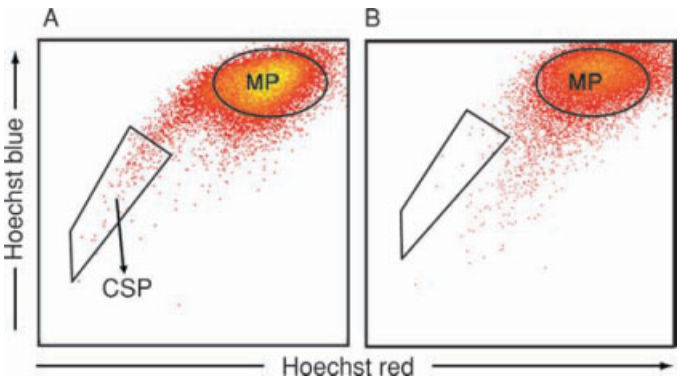


Figure 7.1 Phenotypic characteristic of cardiac side population (CSP) cells. (A) FACS analysis of a cardiomyocyte-depleted nucleated cell suspension following isolation from adult mouse hearts and staining with Hoechst 33342 dye, demonstrating distinct Hoechst extruding CSP and nonextruding main population (MP) cells. (B) FACS analysis following treatment with the ABC transporter inhibitor, fumitremorgin C, demonstrating inhibition of Hoechst efflux from CSP cells.

SP cells [15]. Future studies are needed to definitely determine the developmental origin of cardiac SP cells.

Gene expression profiling of cardiac SP cells

In order to better define the transcriptional profile of cardiac SP cells, Martin et al. performed global gene expression array studies on isolated cardiac SP cells from postnatal murine hearts in comparison with embryonic stem cells, bone-marrow-derived SP cells, and adult cardiomyocytes [12]. This analysis revealed a common molecular program between cardiac SP cells and embryonic stem cells involving the Notch and TGFβ signaling pathways as well as cell cycle regulatory proteins. Moreover, cardiac SP cells were shown to exhibit induction of protective gene programs involving stress pathways, detoxification, and oxidative stress. As expected, cardiac SP cells exhibited profound expression of known molecular determinants of the SP phenotype, the ABC transporter Abcg2, and Mdr1, similar to that demonstrated in bone marrow SP cells [12]. Transcriptional profiling studies further revealed upregulation of many transcriptional regulators in cardiac SP cells, including the MDS box transcription factors of the MEF2 family, which are crucial for cardiomyogenesis [12]. Results from our laboratory have demonstrated expression of early cardiac and endothelial transcription factors in freshly isolated cardiac SP cells, without expression of mature markers of cardiomyogenesis, including myofilament structural genes [14]. Taken together, these genetic profiling studies suggest that cardiac SP cells represent primitive cells, which share common gene programs with embryonic and hematopoietic stem cells but lack markers specific for cardiac differentiation [12, 14]. Also, notably, cardiac SP cells share many gene expression features with previously well-characterized c-kit+/lin− cardiac progenitor cells described by Anversa and colleagues [20], including expression of c-kit, Sca-1, and Mdr1 genes. However, in contrast to c-kit+/lin− cardiac progenitor cells, which are specifically purified according to their c-kit antigen cell surface expression, cardiac SP cells exhibit limited c-kit expression, as assessed by FACS analysis, though this may be due to enzymatic cleavage during the preparation of the cell suspensions before Hoechst staining [12, 14].

Differentiation potential of cardiac SP cells

Tissue-derived SP cells in the liver, skeletal muscle, mammary gland, and testis have all been shown to differentiate into cell lineages of their harboring organ [10]. With the recent identification of SP cells in the myocardium, much attention has been dedicated to the investigation of the cardiomyogenic potential of cardiac SP cells. Hierlihy et al. and Martin et al. first reported expression of connexin 43 and α-sarcomeric actinin, respectively, in cardiac SP cells cocultured with other cardiac cells [11, 12]. Our results demonstrate the capacity for biochemical and, more importantly, functional cardiomyogenic differentiation in cardiac SP cells [14]. Notably, our study demonstrated that among cardiac SP cells, cardiomyogenic differentiation is restricted to cells negative for CD31 expression and positive for Sca-1 expression (CD31−/Sca-1+ SP cells) [14]. These cells adhere to laminin-coated culture dishes and robustly expressed proteins of the MEF2 and GATA family—crucial transcriptional regulators for cardiomyogenic differentiation. Such transcription factor expression was shortly followed by expression of cardiac-specific contractile proteins, including α-sarcomeric actinin and troponin I.

Despite such robust cardiac protein expression, cardiac SP cells lacked proper sarcomeric organization and contractile activity indicative of an immature cardiomyogenic phenotype. Additional stimuli mediated through cellular interaction and electromechanical coupling with cardiomyocytes were necessary to promote further maturation of cardiac SP cells [14]. In this *in vitro* system, coupled cardiac SP cells developed full sarcomeric organization, with ordered myofibrils and a clear striation pattern, and exhibited spontaneous as well as stimulated contraction in synchrony with adjacent cardiomyocytes (Figure 7.2). Intracellular calcium measurements in contracting cardiac SP-cell-derived cardiomyogenic cells demonstrated intracellular calcium transients indistinguishable from those seen in cocultured adult cardiomyocytes. Importantly, this differentiation process occurred independently of cellular fusion with cardiomyocytes. These studies

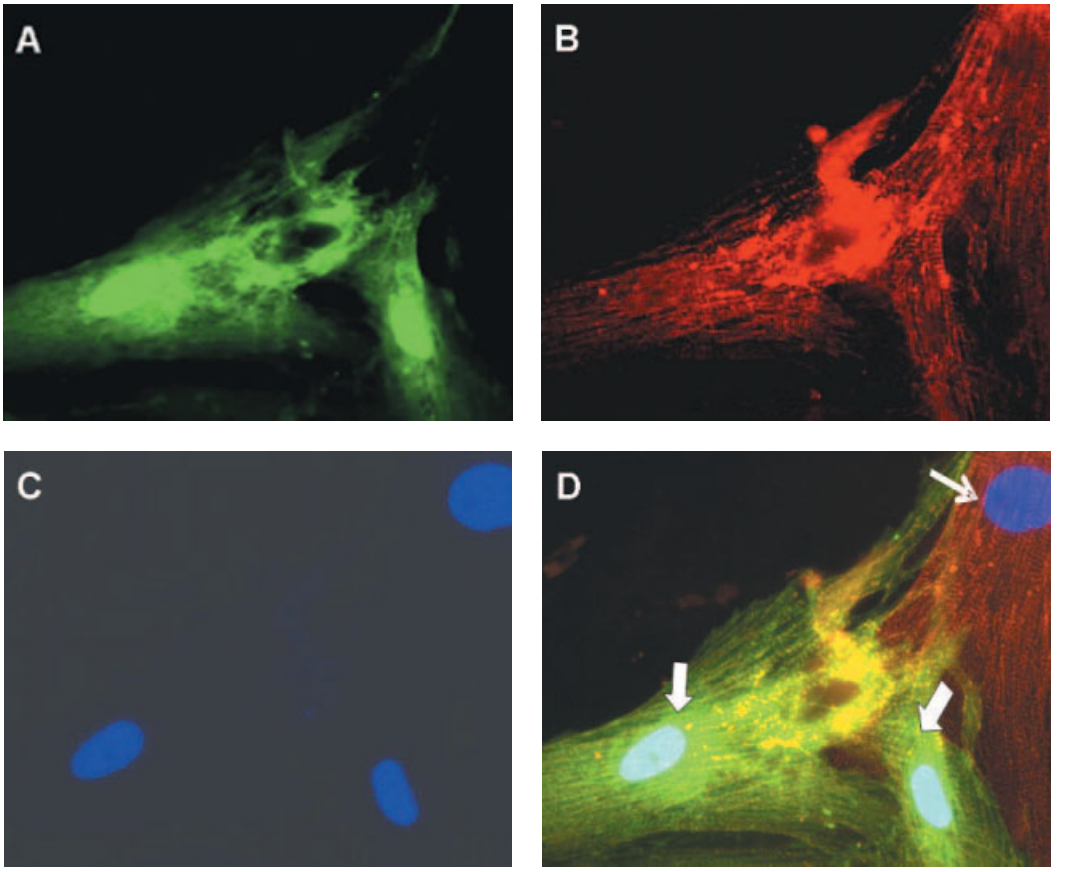


Figure 7.2 Cardiomyogenic differentiation of cardiac SP cells cocultured with adult cardiomyocytes. Fluorescence images of (A) GFP-expressing cardiac SP cells (green) cocultured with adult cardiomyocytes, (B) with immunofluorescent detection of α-actinin (red), and (C) with DAPI nuclear costain (blue). (D) Merged images show areas of colocalization (yellow). The large arrowheads point to two cardiomyogenic differentiated cardiac SP cells with clearly organized sarcomeric structures, while the small arrow points to a cocultured adult cardiomyocyte.

demonstrated for the first time that CD31−/Sca-1+ cardiac SP cells isolated from postnatal murine hearts have the capacity for differentiation into functional cardiomyocytes. In contrast, cardiac SP cells expressing the endothelial marker CD31 failed to undergo cardiomyogenic differentiation, but differentiated into endothelial cells if cultured in endothelial cell growth promoting media, suggesting that these cells were already committed to the endothelial cell lineage (O. Pfister and R. Liao, unpublished data).

Recent studies by Tomita and colleagues demonstrated the generation of neurosphere-like clusters, referred to as *cardiospheres*, from neonatal cardiac SP cells [15]. Similar to the cardiospheres described by Messina et al. [21], cardiospheres derived from cardiac SP cells have been shown to harbor clonogenic cells with remarkable multilineage differentiation potential [15]. Dissociated cells from such cardiospheres exhibited expression of cardiac, smooth muscle, and neuronal genes and proteins, thus further supporting the assumption that cardiac SP cells might derive from the cardiac neural crest. While the *in vitro* cardiomyogenic differentiation potential of cardiac SP cells has been consistently demonstrated by various groups, less is known about the capacity of these cells to generate cardiomyocytes *in vivo*. Recently, Komuro and colleagues studied the homing and differentiation efficiency of intravenously injected cardiac-derived

cells in a myocardial cryoinjury rat model [22]. In this study, neonatal rat cardiac SP cells were shown to home with much higher efficiency into the injured myocardium than cardiac cells lacking the SP phenotype. Weeks after intravenous cell injection, homed cardiac SP cells showed robust expression of GATA4, α-sarcomeric actinin, von Willebrand factor, smooth muscle actin, and vimentin, suggestive of their differentiation into cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts [22]. In contrast, injected non-SP cells lacked expression of cardiomyogenic markers. Although these results support the notion of cardiomyogenic differentiation of cardiac SP cells *in vivo*, further *in vivo* studies are warranted to thoroughly characterize cardiac SP cells following their implantation into the adult myocardium.

Functional significance of cardiac SP cells

As the SP phenotype is defined by the ability to efflux Hoechst dye *in vitro* rather than by a specific molecular marker, identification of SP cells *in situ* remains technically difficult, limiting cell tracking and functional studies of cardiac SP cells *in vivo*. To date, investigation of SP cell function *in vivo* has been limited to labeled transplantation experiments, in which defined populations of labeled SP cells have been reintroduced into animals to study their fate and function. Such transplantation experiments in cardio-injured animals consistently demonstrate high affinity of cardiac SP cells to injured myocardium. Injection of labeled cardiac SP cells into the remote, noninjured myocardium demonstrated migration of labeled cells from the remote area to the infarct border zone (F. Mouquet and R. Liao, unpublished data), similar to previously labeled c-kit+ cardiac progenitor cells [23]. Moreover, intravenously injected GFP+ cardiac SP cells specifically home to areas of injured myocardium, and express cardiac-specific proteins [22], raising the possibility for myocardial repair.

In order to more specifically delineate the response of endogenous cardiac SP cells to myocardial injury, we recently performed a serial assessment of cardiac SP pools in mice hearts following myocardial infarction [19]. Results obtained from this study demonstrated that cardiac SP cells are acutely depleted after myocardial infarction, most likely because of necrotic cell death within the infarct area. Notably, however, their numbers recovered to preinjury levels within 7 days after acute myocardial infarction. This rapid reconstitution of cardiac SP cell pools involved an injury-triggered activation of endogenous cardiac SP cell proliferation as well as myocardial homing of bone-marrow-derived SP cells. Activation of the endogenous cell proliferation was evidenced by an increase of Ki67+ cardiac SP cells 3 days following myocardial infarction. The homing of bone marrow-derived SP cells was demonstrated by the detection of GFP+ cardiac SP cells following myocardial infarction in the GFP bone-marrow-transplanted mice. Also, immunophenotypic characterization of bone-marrow-derived SP cells, following migration to injured myocardium, demonstrated a phenotypic shift with loss of CD45 expression, suggesting a cardiac-niche-dependent conversion of bone marrow SP cells to cardiac SP cells [19]. It remains to be determined whether such bone marrow-derived cardiac SP cells exhibit the same functional properties as their endogenous counterparts, or whether their capacity for cardiomyogenic differentiation is reduced because of their noncardiac origin.

The stem cell-like transcriptional gene profile, the ability to differentiate into functional cardiomyocytes *in vitro*, as well as the activation, migration, and homing of cardiac SP cells into areas of injury after myocardial infarction strongly suggest that cardiac SP cells represent a cardiac progenitor population involved in cardiac homeostasis and repair. Importantly, cardiac SP cells express the ABC transporters Abcg2 and Mdr1 [12], both of which are involved in promoting cell survival and protection from environmental stressors, particularly under hypoxic conditions [24, 25]. Abcg2 is regulated by the hypoxia-inducible transcription factor complex, emphasizing the particular role of this molecule in hypoxia. Recent studies demonstrated that primitive cells lacking Abcg2 exhibited poor survival under low-oxygen condition because of the deleterious accumulation of heme degradation products such as porphyrins [26]. Indeed, porphyrins were shown to represent specific substrates of the Abcg2 transporter, suggesting that this transporter is crucial for the maintenance of

porphyrin homeostasis in progenitor cells exposed to hypoxia [26]. Increased expression of the cell surface P-glycoprotein encoded by the *Mdr1* gene is best known for its ability to provide intrinsic and acquired multidrug resistance in various cancer cells. In addition to its capacity to efflux cytotoxic substrates, Mdr1 has also been shown to play a fundamental role in promoting cell survival by conferring resistance to apoptotic stimuli exerted by Fas ligand and tumor necrosis factor. By inhibiting the activation of caspases 3 and 8, downstream targets of the apoptotic cascade, Mdr1 prevents the final execution of apoptotic cell death [27]. Moreover, Mdr1 also provides protection from cell lysis induced by activated complement [28]. Such protection mechanisms from a wide range of cytotoxic stimuli are crucial to enable the long-term survival of cardiac progenitor cells. Particularly after ischemia/reperfusion injury, myocardial cells are subjected to increased apoptosis and complement activation. The increased ABC transporter activity present in cardiac SP cells may therefore protect these progenitor cells against such an adverse local environment. Moreover, expression of a wide panel of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase enables cardiac SP cells to maintain intracellular redox balance even under conditions of increased oxidative stress [12], which also is present in the myocardium following ischemia.

Further *in vivo* studies are needed to address the functional significance of cardiac SP cells in cardiac homeostasis and repair. In particular, novel methods for tracking cardiac SP cells *in vivo* are required to fully understand their role in cardiac repair following myocardial injury. Furthermore, whether expression of prosurvival transporters and *in vivo* differentiation of cardiac SP cells translates into functional improvement of remodeling and cardiac performance following myocardial infarction has yet to be investigated thoroughly, particularly in comparison with the transplantation of non-SP cells.

Conclusion and outlook

Resident progenitor cells, e.g. cardiac SP, can be purified according to their increased Hoechst efflux capacity using FACS analysis. These cardiac SP cells are enriched in cardiac progenitor cell activity, exhibiting both self-renewal as well as the capacity for differentiation into functional cardiomyocytes, endothelial cells, and smooth muscle cells, both *in vitro* and *in vivo* following myocardial injury. As Hoechst dye efflux is an active biological process, cardiac SP cells represent a relatively heterogeneous cell population compared to other putative progenitor cells isolated by a definitive cell surface profile. Future studies on cardiac SP cells will help to further define specific molecular markers for identification and tracking of SP cells, to identify subpopulations or cardiac SP cells further enriched for differentiation, and to specifically address the functional significance of the ABC transporter system in cardiac progenitor cell biology. Also, future studies are required to effectively expand isolated cardiac SP cells *in vitro* and to determine the functional significance of both endogenous and implanted cardiac SP cells on myocardial homeostasis and repair. While our understanding of cardiac SP cells remains incomplete, recent work has raised the potential of harnessing these cells for cardiovascular repair.

Acknowledgments

This work was supported by research grants funding from the National Institutes of Health (R.L.). O.P. is a recipient of a Swiss National Science Foundation Grant (PBBSB-101349) and F.M. is a recipient of a Federation Francaise de Cardiologie Grant. Fumitremorgin C is a generous gift from Dr. Richard C. Mulligan at the Harvard Gene Therapy Initiative, Harvard Medical School.

References

1 Lakshmipathy U, Verfaillie C. Stem cell plasticity. Blood Rev 2005; 19:29–38.
2 Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988; 241:58–62.
3 Wolf NS, Kone A, Priestley GV, Bartelmez SH. In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp Hematol 1993; 21:614–622.
4 Schinkel AH, Mayer U, Wagenaar E, et al. Normal viability and altered pharmacokinetics in mice lacking mdr1-type

(drug-transporting) P-glycoproteins. Proc Natl Acad Sci USA 1997; 94:4028–4033.
5 Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001; 7:1028–1034.
6 Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 2002; 20:11–20.
7 Goodell MA, Brose K, Paradis G, et al. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996; 183:1797–1806.
8 Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 2002; 99:507–512.
9 Asakura A, Rudnicki MA. Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp Hematol 2002; 30:1339–1345.
10 Challen GA, Little MH. A side order of stem cells: the SP phenotype. Stem Cells 2006; 24:3–12.
11 Hierlihy AM, Seale P, Lobe CG, et al. The post-natal heart contains a myocardial stem cell population. FEBS Lett 2002; 530:239–243.
12 Martin CM, Meeson AP, Robertson SM, et al. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 2004; 265:262–275.
13 Oh H, Bradfute SB, Gallardo TD, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 2003; 100:12313–12318.
14 Pfister O, Mouquet F, Jain M, et al. CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 2005; 97: 52–61.
15 Tomita Y, Matsumura K, Wakamatsu Y, et al. Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol 2005; 170:1135–1146.
16 Kotton DN, Fabian AJ, Mulligan RC. A novel stem-cell population in adult liver with potent hematopoietic-reconstitution activity. Blood 2005; 106:1574–1580.
17 Summer R, Kotton DN, Liang S, et al. Embryonic lung side population cells are hematopoietic and vascular precursors. Am J Respir Cell Mol Biol 2005; 33:32–40.
18 Summer R, Kotton DN, Sun X, et al. Translational physiology: origin and phenotype of lung side population cells. Am J Physiol Lung Cell Mol Physiol 2004; 287:L477–L483.
19 Mouquet F, Pfister O, Jain M, et al. Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. Circ Res 2005; 97:1090–1092.
20 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.
21 Messina E, DeAngelis L, Frati G, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004; 95:911–921.
22 Wada H, Matsuura K, Oyama T, et al. Homing and differentiation of intravenously transplanted cardiac side population cells in injured myocardium. Circulation 2005; 111:801.
23 Urbanek K, Torella D, Sheikh F, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 2005; 102:8692–8697.
24 Comerford KM, Wallace TJ, Karhausen J, et al. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 2002; 62:3387–3394.
25 Krishnamurthy P, Schuetz JD. Role of abcg2/bcrp in biology and medicine. Annu Rev Pharmacol Toxicol 2006; 46:381–410.
26 Krishnamurthy P, Ross DD, Nakanishi T, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem 2004; 279:24218–24225.
27 Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci USA 1998; 95:7024–7029.
28 Weisburg JH, Roepe PD, Dzekunov S, Scheinberg DA. Intracellular pH and multidrug resistance regulate complement-mediated cytotoxicity of nucleated human cells. J Biol Chem 1999; 274:10877–10888.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

8

CHAPTER 8

Phenotype and function of c-kit$^+$-derived amplifying myocytes

Rachel Wilson, BS, *Xiongwen Chen,* PhD, *Hajime Kubo,* PhD, *Remus Berretta,* BS, *& Steven R. Houser,* PhD

Introduction

In the nineteenth century the basis of cardiac enlargement during normal postnatal growth and in response to pathological cardiovascular stress was thought to involve both hypertrophy (myocyte enlargement) and hyperplasia (an increase in myocyte number via cellular proliferation). By the twentieth century, however, the view that new myocytes could be generated in the postnatal heart was disregarded by most investigators because of their inability to observe mitotic figures in intact myocardium and the inability to induce myocyte proliferation *in vitro*. Thus, for most of the last century, the heart has been considered a terminally differentiated organ composed of myocytes that lose their ability to divide shortly after birth [1]. This scenario leaves the postnatal myocardium with a limited capacity to respond to both physiological and pathological stressors that require an increase in cardiac mass.

The recent discovery of a population of resident cardiac stem cells (CSCs) with the capacity to differentiate into cardiac myocytes challenges the view that the heart is a terminally differentiated organ with no capacity for new myocyte generation. A number of recent studies from several different laboratories show that stem cells capable of differentiating into functional cardiac myocytes are resident in the heart [2–4] or can be derived from hematopoietic precursors [5–7]. The fact that new myocytes can be formed from resident CSCs is consistent with the hypothesis that these cells are part of a homeostatic process for endogenous repair or maintenance of the normal heart.

In recent studies from our group, reviewed in this chapter, we have examined the idea that new myocyte formation contributes to adolescent heart growth. Increases in cardiac mass in response to hemodynamic stress have long been considered the exclusive result of myocyte hypertrophy and interstitial proliferation, but recent studies have reasserted the postulate that this cardiac enlargement results from both cellular hypertrophy and hyperplasia [8–10]. These studies of diseased hearts will not be reviewed further here. Our studies with adolescent large mammals have examined the idea that the young adult heart has the capacity to generate new myocytes from a pool of resident c-kit$^+$ stem cells and that the period of rapid cardiac growth that precedes sexual maturity involves an increase in myocyte number. In these studies we have identified newly formed cardiac myocytes and have measured their physiological properties. In this chapter we review the evolving evidence from our laboratory and from other groups that supports the idea that new myocytes, with unique physiological properties, can be derived from resident c-kit$^+$ CSCs. We compare and contrast the properties of these newly formed adult myocytes to neonatal and adult cardiac myocytes.

Stem cells can differentiate into cardiac myocytes

A number of studies have shown that stem cells from a variety of sources have the capacity to differentiate into cells expressing cardiac

myocyte-specific proteins. CSCs, mesenchymal stem cells, hematopoietic stem cells, and blood-derived endothelial progenitor cells from humans [11, 12] and rodents [13–15] can differentiate into cells expressing cardiomyocyte-specific proteins, especially when cocultured with neonatal cardiac myocytes. Most of these studies have used staining techniques to identify differentiated cell types and have not characterized the physiological properties of the newly formed, stem cell-derived myocytes. Only a few studies have examined the functional properties of these cells. Human endothelial progenitor cells that were cocultured with neonatal rat myocytes for 6 days formed gap junctions with the neonatal cells and exhibited calcium transients similar to and synchronized with those in the neonatal rat myocytes [11]. Stem cell antigen-1 positive (Sca-1^+) cells isolated from the adult mouse heart and cocultured with adult rat myocytes demonstrate gap junction formation with the cocultured adult myocytes, and respond to electrical stimulation with cellular contractions and calcium transients indistinguishable from the adult myocytes [15]. Human embryonic stem cells can form spontaneously beating cardiac myocytes *in vitro*, with stimulated contractions and action potentials similar to those seen in the embryonic heart [16]. These studies show that several types of stem cells have the capacity to differentiate into cells with cardiac myocyte properties when cocultured with neonatal myocytes, although some studies have not been able to induce stem cells to differentiate into functional myocytes with these approaches [17]. The cellular and molecular bases for the differentiation and/or proliferation of a stem cell into a cardiac myocyte in the presence of a neonatal myocyte are yet to be determined. Clearly, CSCs and other stem cell types can differentiate into cells that express cardiac myocyte proteins in the absence of neonatal myocyte coculture [4]. However, these cells appear to be functionally less competent (P. Anversa, personal communication). One study suggests that contact between islet-1-expressing cardiac cells and fixed neonatal myocytes is sufficient to induce stem cell differentiation [18], but this result is yet to be confirmed by others.

What seems clear from the studies performed to date is that coculturing stem cells with functional neonatal myocytes promotes differentiation of these cells into cardiac myocytes. While the phenotype of these myocytes has been examined, no clear picture of the type of cardiac myocyte that develops has evolved. The expectation that the stem cell-derived cardiac myocyte in coculture with neonatal cardiac myocytes, would have properties like those of an adult myocyte seems unrealistic. It is well known that adult myocytes change their structure and function in primary culture, so even they do not have the properties they exhibit *in vivo* [19]. Therefore, the properties of stem cell-derived cardiac myocytes in culture are not easily predicted, but are not expected to be like those of myocytes freshly isolated from the normal adult myocardium. However, if these stem cell-derived cardiac myocytes are at low density and are electrically coupled to neonatal myocytes, they will have resting and action potentials similar to those of the neonatal myocytes to which they are connected. This would be the case even if the newly formed myocytes expressed ion channels and Ca^{2+} regulatory proteins distinct from those of the neonatal myocytes. Newly formed myocytes in coculture with neonatal myocytes should have basal electrical properties similar to neonatal myocytes, including a less well-polarized resting potential, spontaneous membrane depolarization (and beating), and action potentials with long duration plateau phases [20–22]. The types of ion channels and Ca^{2+} regulatory proteins expressed by these new myocytes have not yet been fully characterized, but it seems clear that these cells will not have properties of mature adult ventricular myocytes, since adult myocytes in primary culture revert to a less well-differentiated state as mentioned earlier. The studies performed to date show that a variety of stem cells have the capacity to differentiate into cardiac myocytes and that the environment generated by neonatal myocytes in primary culture promotes this differentiation by processes that are yet to be fully determined. This neonatal coculture system will be useful for studies that seek to identify those factors that regulate stem cell differentiation into a variety of cardiac cell types.

Feline c-kit$^+$ cardiac stem cells

During adolescence (age 3–8 months in felines), mammals experience a rapid growth phase during which both their body and heart weights rapidly

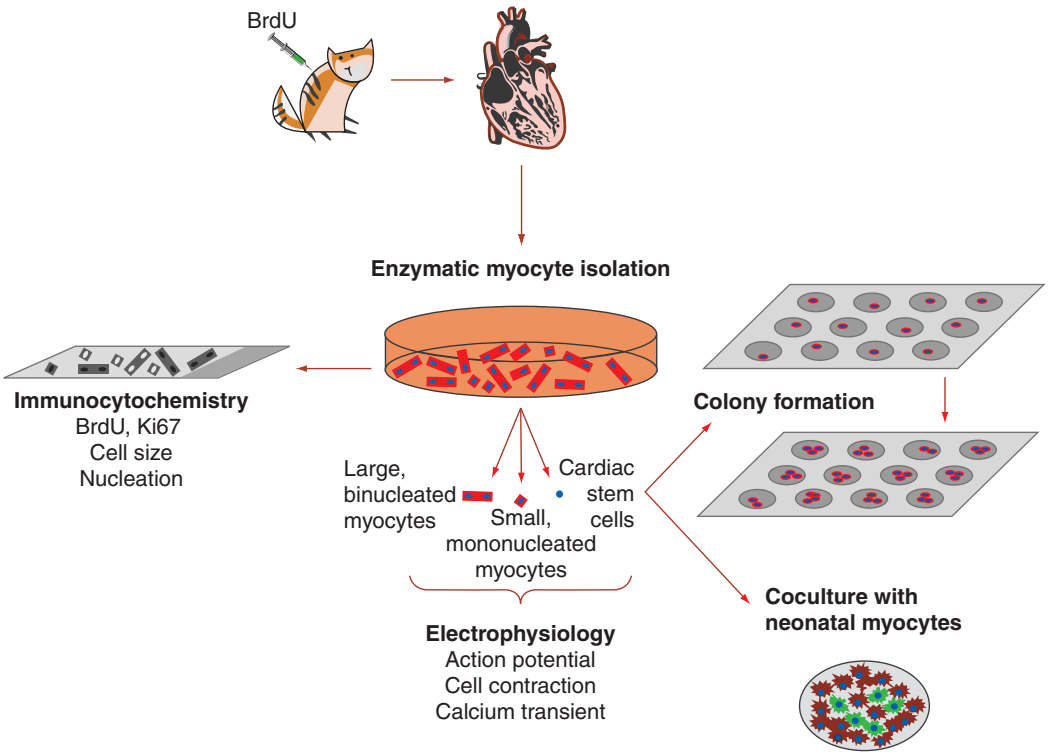


Figure 8.1 The experimental design of our studies to explore the hypothesis that c-kit+ cardiac stem cells differentiate into new cardiac myocytes. Adolescent male cats received a constant infusion of BrdU (10 mg/(kg day)) for 7 days via osmotic minipumps. The heart was then excised and enzymatically dissociated. Differential centrifugation and filtration followed by magnetic cell sorting was used to separate c-kit+ small cells from myocytes. c-kit+ cells were plated individually and used for clonal colony formation or cocultured (after infection with GFP-containing adenovirus) with neonatal rat myocytes. Isolated feline myocytes were either used for electrophysiological and functional measurements (action potential, myocyte contraction, calcium transients, calcium currents) or fixed and used for immunocytochemistry (BrdU, Ki67).

increase. We used enzymatic dissociation techniques to isolate myocytes and CSCs from the hearts of felines in this age range (Figure 8.1). Using magnetic cell sorting, we obtained a population of small c-kit+ (a stem cell marker) stem cells from these hearts and used limiting dilution techniques to culture single c-kit+ cells. These single c-kit+ CSCs were clonogenic, self-renewing, and capable of differentiating into cells expressing cardiac myocyte-specific transcription factors (myocyte-enhancing factor 2c) and proteins (cardiac actin). However, these cells neither beat spontaneously nor respond to field stimulation under the conditions we have tested to date. Therefore, we placed these c-kit+ CSCs into coculture with neonatal rat ventricular myocytes. c-kit+ CSCs were infected with an adenovirus containing green fluorescence protein (GFP) so that these cells could be identified when placed in coculture with unlabeled neonatal rat ventricular myocytes. These GFP-expressing cells differentiated into beating cells that expressed cardiac-specific proteins (α-sarcomeric actin), and these new cardiac myocytes were electrically coupled to the neonatal myocyte feeder layer via gap junctions, demonstrated by connexin 43 staining. Newly formed c-kit+-derived cardiac myocytes had spontaneous action potentials, and electrical stimulation also evoked action potentials at different rates. The evoked and spontaneous action potentials were similar to those of the neonatal myocytes to which these cells were electrically coupled. When exposed to the β-adrenergic agonist isoproterenol, the c-kit+-derived cardiac myocytes had an increased beating rate and an increase in the size of their

cytosolic Ca^{2+} transient. These studies show that the adolescent feline heart contains a resident population of c-kit$^+$ stem cells that have the ability to self-renew and differentiate into functionally competent new cardiac myocytes, with the capacity to integrate (electrically couple) with neighboring myocytes. Our next step was to explore the idea that functionally competent new myocytes from c-kit$^+$ CSCs are formed *in vivo*, under normal physiological conditions. We specifically studied this idea in the adolescent heart, during a time when heart size is rapidly increasing.

New myocyte formation in the adolescent feline heart

We are currently testing the hypothesis that new myocytes are generated from c-kit$^+$ CSCs in the adolescent feline heart. This hypothesis predicts that newly formed myocytes should initially be small and mononucleated. Therefore, we characterized the size of mono- and binucleated feline myocytes and their respective percentages of total ventricular myocytes. The majority of myocytes in the adolescent feline left ventricle were binucleated (88.2 ± 4.1% binucleated, 10.5 ± 4.7% mononucleated; $n = 6$ hearts, $n = \geq 1000$ myocytes per heart). The binucleated cells were significantly larger than the mononucleated cells (two-dimensional surface area was 2589 ± 342 μm^2 and 1559 ± 207 μm^2, respectively, $n = 150$ myocytes per heart, $p = 0.01$). In rats and mice, late-gestation cardiac myocytes are almost exclusively mononucleated, but shortly after birth become binucleated [8, 23, 24], so that by 2 weeks of age, more than 95% of cardiac myocytes are binucleated. This change from mono- to binucleation correlates with the transition from myocyte hyperplasia to hypertrophic growth [24, 25]. The fact that mononucleated myocytes make up a considerable percentage of the adolescent myocardium and are significantly smaller than the binucleated myocytes supports the idea that at least some small mononucleated myocytes are newly derived from a stem cell pool. The fact that we have been able to show that these cells express proteins associated with the cell cycle, including Ki67 and Brdll, also supports the working hypothesis.

5-Bromo-2-deoxyuridine (BrdU) is a thymidine analog that incorporates into DNA during the S phase of the cell cycle and is commonly used as an index of cell cycle activity and new cell formation. We measured BrdU incorporation into cardiac myocytes after 1 week of constant subcutaneous BrdU infusion (10 mg/kg day). After isolating ventricular myocytes from these hearts, we found that approximately 1.2% of all left ventricular myocytes were positive for BrdU. The majority of BrdU incorporation, moreover, was in small, mononucleated myocytes (Table 8.1). These data were supported by immunostaining for Ki67, a nuclear protein expressed in all phases of the cell cycle except G_0. Ki67 staining was also found predominantly in small, mononucleated myocytes. These data support our hypothesis that new myocytes are generated from c-kit$^+$ CSCs during adolescent cardiac growth and strongly support the idea that new myocytes are formed in the normal heart after birth. Importantly, a number of other laboratories have reported BrdU labeled myocytes in young animals as well (for review, see [26]). Collectively, these studies suggest that young animals have the capacity to enlarge their hearts through a combination of growth (hypertrophy) of existing myocytes and the generation of newly formed myocytes, in all likelihood from a resident CSC pool.

Table 8.1 Myocyte BrdU incorporation.

Animal age (d)	*Heart wt (g)*	*Mononucleated (%)*	*Mononucleated BrdU$^+$ (%)*	*Binucleated (%)*	*Binucleated BrdU$^+$ (%)*	*Total BrdU$^+$ (%)*
171 ± 4.4	11.96 ± 0.71	10.7 ± 2.5	4.84 ± 3.39	88.0 ± 2.2	0.73 ± 0.38	1.20 ± 0.77

After 1 week of constant BrdU infusion (10 mg/(kg day)), left ventricular (LV) myocytes were isolated and stained for BrdU. The majority of myocytes in the LV are binucleated, and a disproportionate percentage of the mononucleated myocytes were BrdU$^+$. Data presented as mean ± SD. $N = 3$ hearts; $n \geq 1000$ myocytes/heart.

Physiological phenotype of newly formed ventricular myocytes

Small, mononucleated myocytes are a distinct subpopulation of cardiac myocytes that preferentially incorporate BrdU and express cell cycle proteins. We have tested the hypothesis that these cells have electrophysiological and functional properties that differ from the large, binucleated ventricular myocytes that compose the majority of the adolescent heart. Importantly, our previous studies have shown that myocyte physiological function is not dependent on the size of the ventricular myocyte [27]. Over the past 25 years, we have characterized the contractile, electrophysiological and Ca^{2+} regulatory phenotype of feline and human myocytes over a broad range of myocyte sizes. These studies show that over the range of relatively large cell sizes used in these studies, myocyte functional phenotype is size independent. These results are consistent with those of many others that show that the functional unit of the cell, the sarcomere, is identical from region to region within the cell [28]. As normal cells enlarge, they simply build more units (sarcomeres). Therefore, our studies and many others show that the unit function of the cardiac myocyte is identical in cells of different size. This is not to imply that cell function never changes, since we and many others have shown that myocyte function is altered in myocytes with hypertrophy and failure [29–31]. These changes, however, are not due to the enlargement of the myocytes but rather represent a change in the unit function of the hypertrophied/failing cardiac myocytes. In our current studies, we are asking a somewhat different question: do small, c-kit$^+$ stem cell-derived cardiac myocytes exist in the normal heart and do they have different functional properties (per unit of myocardium) than the other myocytes in the heart?

The vital nuclear dye Hoechst 33258 can be used to identify mono- and binucleated ventricular myocytes that can then be functionally phenotyped. We first examined myocyte contractile properties in the smallest mononucleated myocytes (SMMs; average length 70 μm) isolated from the adolescent feline heart and compared them to the large binucleated myocytes (LBMs; average length 110 μm) that are the vast majority of myocytes in these hearts. In myocytes stimulated at 0.5 Hz, the magnitude of fractional shortening was similar (approximately 8%) between the two groups, but the maximal rates of contraction and relaxation were significantly slower in SMMs and the duration of contraction was longer. These results show that the smallest myocytes in the normal adolescent heart have fundamentally different contractile properties than those of the majority of the myocytes.

Cardiac myocyte contraction is dependent on the rate of rise, magnitude, and duration of the intracellular Ca^{2+} transient. We have determined that the differences in contraction between SMM and LBM groups are due to alterations in Ca^{2+} handling. The amplitude of the Ca^{2+} transient was significantly smaller in SMMs than in LBMs, and the rate of rise of the transient to the peak was also significantly slower in SMMs. This alone would result in a decrease in the Ca^{2+} available to bind the myofilament proteins and induce contraction and would decrease contractility. However, the configuration of the Ca^{2+} transient was also different between the two groups (Figure 8.2). The Ca^{2+}transient of SMMs had both a rapidly rising phase and a slowly rising phase, similar to that seen in neonatal myocytes [32, 33], whereas the LBMs exhibited only the rapidly rising phase. This caused the duration of the Ca^{2+} transient to be significantly longer in SMMs. Prolongation of the Ca^{2+} transient increases the time available for Ca^{2+} binding to myofilament proteins and increases contraction. Finally, the rate of decay of the Ca^{2+} transient was also significantly slower in SMMs than in LBMs.

These studies show that multiple differences in the Ca^{2+} transient exist between SMMs and the majority of myocytes in the normal heart. The determinants of these differences are currently being explored, but are predictable, based on our current understanding of Ca^{2+} regulation in fetal/neonatal, normal adult, and diseased ventricular myocytes [34, 35]. The slow rate of rise likely reflects a less well-developed sarcoplasmic reticulum (SR), with an increased reliance on Ca^{2+} influx for the initiation of contraction. The slower rate of decay of the Ca^{2+} transient in SMMs also suggests a less well-developed SR and a heavier reliance on sarcolemmal Na^+/Ca^{2+} exchange for Ca^{2+} efflux. These differences are similar to those between neonatal and adult cardiac myocytes. Neonatal myocytes have a less well-developed SR than adult myocytes and rely

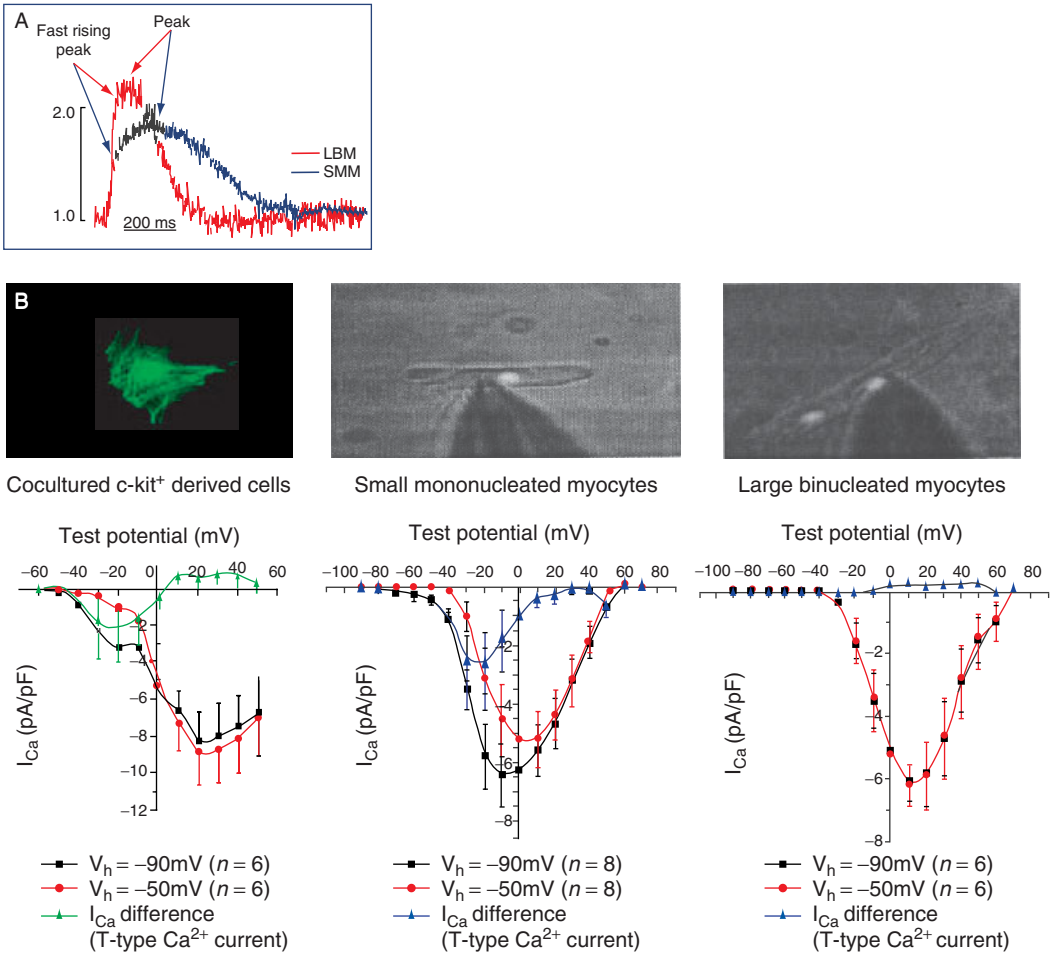


Figure 8.2 Ca^{2+} transients and L- and T-type Ca^{2+} Currens. (A) Representative Ca^{2+} transients from large binucleated (red trace) and small mononucleated (blue trace) myocytes are shown. See text for discussion. (B) Both T-type (I_{Ca-T}) and L-type (I_{Ca-L}) Ca^{2+} currents were present in c-kit^{+}-derived cardiac cells (left panels) and in small mononucleated myocytes (middle panels), but only the L-type Ca^{2+} current was present in large binucleated myocytes (right panels). Error bars represent standard error of the mean.

more heavily on Ca^{2+} influx for activation of contraction [35].

These results show that the smallest ventricular myocytes in the normal adolescent heart have different, more rudimentary, physiological properties than the majority of the myocytes in the heart. We examined the idea that SMMs have a less well-developed SR than LBMs by measuring localized cellular Ca^{2+} release with line scan confocal microscopy, as described in a recent report [36]. These experiments showed that SR Ca^{2+} release was less well organized in SMMs than in LBMs, consistent with a less well-developed SR. Related studies showed that the transverse tubular system, which structurally interacts with SR Ca^{2+} storage and release sites, was also less well developed in SMMs. Together, these results show that SMMs have less "mature" structural and functional properties, consistent with the idea that they are newly derived from a stem cell. These results will need to be confirmed by other groups.

Interestingly, SMMs have some electrophysiological and functional characteristics reminiscent of myocytes isolated from pathologically hypertrophied hearts, as opposed to the LBMs which are more representative of isolated myocytes from

control hearts [36–38], a possibility worthy of further study. This may reflect the reexpression of a fetal gene profile in hypertrophy, reported by several labs [39, 40], a possibility worthy of further study.

Ca^{2+} channels in small mononucleated myocytes

Ca^{2+} influx through voltage regulated ion channels is essential to the function of cardiac myocytes. Interestingly, there are distinct Ca^{2+} channels that appear to serve different physiological functions. The best studied of these is the L-type Ca^{2+} channel (LTCC), which is essential in cardiac myocytes [41]. It is expressed from the time of the first heartbeat during embryologic development through adult life. It is critical to the Ca^{2+}-induced Ca^{2+} release process and contraction of cardiac muscle cells. We measured the density and properties of LTCC in SMMs. LTCC density was not different between SMMs and LBMs, which was not surprising since the density of this channel in cardiac myocytes is similar in the embryonic heart and the normal adult heart, and even in many diseased states.

Another voltage-dependent calcium channel, the T-type calcium channel (TTCC), has also been observed in cardiac myocytes. The functional role of Ca^{2+} influx through this channel is still unresolved. We do know, however, that the TTCC plays almost no role in excitation–contraction coupling. This suggests that it may be involved in activation or regulation of Ca^{2+}-activated signaling pathways. The channel is encoded by three different genes ($\alpha_{1,G,H,I}$), two of which (G and H) are expressed in the heart. The TTCC is primarily expressed in fetal and neonatal cardiac myocytes [42–44] and disappears from ventricular myocytes soon after birth. The loss of T-type Ca^{2+} current ($I_{Ca\text{-}T}$) in these cells is associated with their withdrawal from the cell cycle. In a previous study, we did not observe I_{Ca-T} in normal feline ventricular myocytes [45], but we found that the TTCC was reexpressed in some ventricular myocytes after pressure overload-induced cardiac hypertrophy. We suggested that the reexpression of these channels was related to the activation of cardiac hypertrophic signaling pathways. Our current hypothesis is that the TTCC plays a critical role in myocyte differentiation (from CSC precursors) or and proliferation of cardiac myocyte progenitor cells or early committed cells. I_{Ca-T} plays an important role in the differentiation and proliferation of many cell types, including blastocyst-derived stem cells, fibroblasts, vascular smooth muscle cells, endothelial cells, neuronal cells, and neonatal ventricular myocytes [43, 46–50]. Blocking I_{ca-T} inhibits proliferation and differentiation of endothelial cells, smooth muscle cells, and neuronal cells [46, 49]. Our recent studies of SMMs show that I_{Ca-T} is present in most of these cells (Figure 8.2), but is not present in LBMs. Interestingly, I_{Ca-T} was also found in c-kit$^+$-derived cardiac myocytes in coculture with neonatal rat ventricular myocytes (Figure 8.2). These studies suggest that Ca^{2+} influx through the TTCC is centrally involved in the generation of new cardiac myocytes from resident CSCs.

Summary

In summary, the adolescent feline heart contains resident c-kit$^+$ CSCs that are multipotent and clonogenic. These cells have the capacity to differentiate into functionally competent cardiac myocytes in culture. *In vivo*, the adolescent heart has a population of SMMs that incorporate BrdU and express proteins associated with cell cycle activity. These SMMs have a physiological phenotype that suggests that they are not yet fully mature, and they express a Ca^{2+} channel that is associated with cellular proliferation. Future studies will need to confirm these findings and examine the contribution of myocyte regeneration in normal and diseased adult hearts.

References

1 Kajstura J. Myocyte proliferation in heart failure. In Crescenzi M (ed.): Reactivation of the Cell Cycle in Terminally Differentiated Cells. Georgetown, TX: Eurekah.com/Landes Bioscience, 1–10, 2002.

2 Oh H, Bradfute SB, Gallardo TD, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 2003; 100(21):12313–12318.

3 Matsuura K, Nagai T, Nishigaki N, et al. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004; 279(12):11384–11391.

4 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114(6):763–776.

5 Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999; 103(5):697–705.

6 Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107(11):1395–1402.

7 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410(6829):701–705.

8 Olivetti G, Quaini F, Lagrasta C, et al. Myocyte cellular hypertrophy and hyperplasia contribute to ventricular wall remodeling in anemia-induced cardiac hypertrophy in rats. Am J Pathol 1992; 141(1):227–239.

9 Kajstura J, Zhang X, Reiss K, et al. Myocyte cellular hyperplasia and myocyte cellular hypertrophy contribute to chronic ventricular remodeling in coronary artery narrowing-induced cardiomyopathy in rats. Circ Res 1994; 74(3):383–400.

10 Olivetti G, Ricci R, Anversa P. Hyperplasia of myocyte nuclei in long-term cardiac hypertrophy in rats. J Clin Invest 1987; 80(6):1818–1821.

11 Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003; 107(7):1024–1032.

12 Garbade J, Schubert A, Rastan AJ, et al. Fusion of bone marrow-derived stem cells with cardiomyocytes in a heterologous in vitro model. Eur J Cardiothorac Surg 2005; 28(5):685–691.

13 Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol 2006; 109(1):74–81.

14 Eisenberg LM, Burns L, Eisenberg CA. Hematopoietic cells from bone marrow have the potential to differentiate into cardiomyocytes in vitro. Anat Rec A Discov Mol Cell Evol Biol 2003; 274(1):870–882.

15 Pfister O, Mouquet F, Jain M, et al. CD31− but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 2005; 97(1):52–61.

16 He JQ, Ma Y, Lee Y, et al. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res 2003; 93(1):32–39.

17 Lagostena L, Avitabile D, DeFalco E, et al. Electrophysiological properties of mouse bone marrow c-kit+ cells co-cultured onto neonatal cardiac myocytes. Cardiovasc Res 2005; 66(3):482–492.

18 Laugwitz KL, Moretti A, Lam J, et al. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005; 433(7026):647–653.

19 Mitcheson JS, Hancox JC, Levi AJ. Cultured adult cardiac myocytes: future applications, culture methods, morphological and electrophysiological properties. Cardiovasc Res 1998; 39(2):280–300.

20 Maylie JG. Excitation-contraction coupling in neonatal and adult myocardium of cat. Am J Physiol 1982; 242(5):H834–H843.

21 Klitzner T, Friedman WF. Excitation-contraction coupling in developing mammalian myocardium: evidence from voltage clamp studies. Pediatr Res 1988; 23(4):428–432.

22 Wang Y, Xu H, Kumar R, et al. Differences in transient outward current properties between neonatal and adult human atrial myocytes. J Mol Cell Cardiol 2003; 35(9):1083–1092.

23 Anversa P, Olivetti G, Loud AV. Morphometric study of early postnatal development in the left and right ventricular myocardium of the rat. I. Hypertrophy, hyperplasia, and binucleation of myocytes. Circ Res 1980; 46(4):495–502.

24 Li F, Wang X, Capasso JM, et al. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996; 28(8):1737–1746.

25 Perloff JK. Development and regression of increased ventricular mass. Am J Cardiol 1982; 50(3):605–611.

26 Soonpaa MH, Field LJ. Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res 1998; 83(1):15–26.

27 Duthinh V, Houser SR. Contractile properties of single isolated feline ventriculocytes. Am J Physiol 1988; 254(1, Pt 2):H59–H66.

28 Russell B, Motlagh D, Ashley WW. Form follows function: how muscle shape is regulated by work. J Appl Physiol 2000; 88(3):1127–1132.

29 Harding SE, Davia K, Davies CH, et al. From overload to failure: what happens inside the myocyte. Ann Med 1998; 30 (suppl 1):14–23.

30 Bailey BA, Dipla K, Li S, Houser SR. Cellular basis of contractile derangements of hypertrophied feline ventricular myocytes. J Mol Cell Cardiol 1997; 29(7):1823–1835.

31 Houser SR, Piacentino V, III, Mattiello J, et al. Functional properties of failing human ventricular myocytes. Trends Cardiovasc Med 2000; 10(3):101–107.

32 Seki S, Nagashima M, Yamada Y, et al. Fetal and postnatal development of Ca2+ transients and Ca2+ sparks in rat cardiomyocytes. Cardiovasc Res 2003; 58(3):535–548.

33 Huang J, Xu L, Thomas MJ, et al. L-type Ca2+ channel function and expression in neonatal rabbit ventricular myocytes. Am J Physiol Heart Circ Physiol 2006; 290(6):H2267–2276.

34 Bers DM, Guo T. Calcium signaling in cardiac ventricular myocytes. Ann N Y Acad Sci 2005; 1047:86–98.

35 Tohse N, Seki S, Kobayashi T, et al. Development of excitation-contraction coupling in cardiomyocytes. Jpn J Physiol 2004; 54(1):1–6.

36 Harris DM, Mills GD, Chen X, et al. Alterations in early action potential repolarization causes localized failure of sarcoplasmic reticulum Ca^{2+} release. Circ Res 2005; 96(5):543–550.

37 Houser SR. Reduced abundance of transverse tubules and L-type calcium channels: another cause of defective contractility in failing ventricular myocytes. Cardiovasc Res 2001; 49(2):253–256.

38 Houser SR, Piacentino V, III, Weisser J. Abnormalities of calcium cycling in the hypertrophied and failing heart. J Mol Cell Cardiol 2000; 32(9):1595–1607.

39 Colucci WS. Molecular and cellular mechanisms of myocardial failure. Am J Cardiol 1997; 80(11A):15L–25L.

40 Ghatpande S, Goswami S, Mascareno E, et al. Signal transduction and transcriptional adaptation in embryonic heart development and during myocardial hypertrophy. Mol Cell Biochem 1999; 196(1–2): 93–97.

41 Anderson ME. Ca^{2+}-dependent regulation of cardiac L-type Ca^{2+} channels: is a unifying mechanism at hand? J Mol Cell Cardiol 2001; 33(4):639–650.

42 Gaughan JP, Hefner CA, Houser SR. Electrophysiological properties of neonatal rat ventricular myocytes with alpha1-adrenergic-induced hypertrophy. Am J Physiol 1998; 275(2, Pt 2):H577–H590.

43 Guo W, Kamiya K, Kodama I, Toyama J. Cell cycle-related changes in the voltage-gated Ca^{2+} currents in cultured newborn rat ventricular myocytes. J Mol Cell Cardiol 1998; 30(6):1095–1103.

44 Niwa N, Yasui K, Opthof T, et al. Cav3.2 subunit underlies the functional T-type Ca2+ channel in murine hearts during the embryonic period. Am J Physiol Heart Circ Physiol 2004; 286(6):H2257–H2263.

45 Nuss HB, Houser SR. T-type Ca^{2+} current is expressed in hypertrophied adult feline left ventricular myocytes. Circ Res 1993; 73(4):777–782.

46 Chemin J, Nargeot J, Lory P. Neuronal T-type alpha 1H calcium channels induce neuritogenesis and expression of high-voltage-activated calcium channels in the NG108-15 cell line. J Neurosci 2002; 22(16):6856–6862.

47 Chen CF, Corbley MJ, Roberts TM, Hess P. Voltage-sensitive calcium channels in normal and transformed 3T3 fibroblasts. Science 1988; 239(4843):1024–1026.

48 Day ML, Johnson MH, Cook DI. Cell cycle regulation of a T-type calcium current in early mouse embryos. Pflugers Arch 1998; 436(6):834–842.

49 Schmitt R, Clozel JP, Iberg N, Buhler FR. Mibefradil prevents neointima formation after vascular injury in rats. Possible role of the blockade of the T-type voltage-operated calcium channel. Arterioscler Thromb Vasc Biol 1995; 15(8):1161–1165.

50 Triggle DJ. Cardiovascular T-type calcium channels: physiological and pharmacological significance. J Hypertens Suppl 1997; 15(5):S9–S15.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

9

CHAPTER 9

Cardiac stem cells and their niches

Annarosa Leri, MD, *Alessandro Boni,* PhD, *Robert Siggins,* PhD, *Angelo Nascimbene,* MD, *& Toru Hosoda,* MD

Introduction

The heart has been found to be a self-renewing organ characterized by resident cardiac stem cells (CSCs) and early committed cells (ECCs) stored in niches. This novel view of the heart raises the possibility that defects in myocardial homeostasis and ventricular dysfunction occur because of a progressive increase in the number of CSCs–ECCs permanently withdrawn from the cell cycle. The rate of accumulation of old CSCs–ECCs might be greater than the rate of their death and replacement, leading to the formation of senescent niches and organ aging. The alterations in the cellular composition of the niches can be expected to be associated with modifications in the physiological turnover of myocytes so that old, less efficient cells accumulate in the ventricular myocardium. In the healthy heart, a few CSCs may sustain, when the need arises, the entire replacement of cells dictated by the high functional requirements of the myocardium. This may not be the case in the impaired heart, in which a large number of CSCs may be concurrently involved in the replacement of dying cells. A nonefficient CSC compartment, which is reflected by nonfunctionally competent niches, may be responsible for the imbalance between cell growth and cell death and the ultimate negative outcome of cardiac adaptation. This framework may advance our understanding of the biology of the heart and etiology of heart failure.

A new view of cardiac biology

The heart is composed of myocytes that constitute a population of highly specialized cells. The differentiated state of myocytes has to be tightly regulated because the heart would fail if the majority of its parenchymal cells were not involved in contractile activity. According to the old paradigm, the heart survives and exerts its function until death of the organism, with the same or lesser number of cells that are present at birth [1]. This notion was challenged by experimental findings documenting activation of the cell cycle machinery, coupled with BrdU incorporation, Ki67, MCM5, Cdc6, and cyclin B1 expression in myocyte nuclei [2]. Identification of the mitotic spindle and contractile ring during karyokinesis and cytokinesis has demonstrated that a subpopulation of myocytes can replicate [3]. However, mitotic division of mature myocytes would require dedifferentiation with disassembly of most of the myofibrillar apparatus for the cell to traverse the cell cycle—a biological process that is difficult to imagine, but cannot be excluded. This is why the results on myocyte proliferation left unanswered the question concerning the source of cycling myocytes in the normal and pathological heart. The search for the origin of dividing myocytes was unsatisfactory until CSCs–ECCs were recognized in the human heart and subsequently in the heart of dogs, pigs, rats, and mice [4]. CSCs correspond to undifferentiated lineage negative (Lin^-) cells that express the stem cell surface antigens c-kit, MDR1, and Sca-1 in variable combinations. Isolated Lin^-c-kit^+ cells from the rat heart possess the three properties of stemness: clonogenicity, self-renewal, and multipotentiality [5]. An *in vivo* functional assay consisting of the transplantation of clonogenic Lin^-c-kit^+ cells in the infarcted ventricle confirmed the *in vitro* features of this cell population. Migration, engraftment, proliferation, and multilineage differentiation of CSCs replaced the dead tissue with

contracting myocardium. There is a high degree of coexpression of c-kit, MDR1, and Sca-1 on the same CSCs–ECCs. Also, primitive cells sorted for each of these antigens in mice, rats, and dogs show a comparable growth and differentiation behavior. Thus, the heart is not a postmitotic organ but a self-renewing organ that possesses a stem cell compartment [4–6].

The shift in paradigm from a postmitotic organ to a self-renewing organ has changed dramatically our view of the heart and understanding of the fundamental mechanisms regulating myocyte and organ aging. In the last 70 years, the general belief has been that the number of myocytes in the heart is defined at birth, and these cells persist throughout life. There are men and women 100 years old and older and, according to the old paradigm, all of their myocytes would have lived 100 years or more. In other words, the age of the individuals and the age of their myocytes should coincide. According to the new paradigm, the continuous turnover of myocytes results in a heterogeneous cell population consisting of young, adult, old, and senescent myocytes. Data in humans suggest that this subdivision corresponds to cells of different sizes, raising the possibility that the life span of a cell is associated with a progressive increase in volume of the cell. Young, amplifying, dividing myocytes are less than 180 μm^2 in cross-sectional area, adult nondividing cells are 200–250 μm^2, old nondividing $p16^{INK4a}$ negative cells are 300–500 μm^2, and senescent nondividing $p16^{INK4a}$ positive cells are 600–900 μm^2 [7]. Myocyte length, however, remains relatively constant in all these cell categories varying, at most, from 90 to 120 μm. Importantly, young myocytes do not express inhibitors of the cell cycle such as p53 and $p16^{INK4a}$. In contrast, a fraction of these cells is cycling as demonstrated by the nuclear expression of the cell cycle markers Ki67, MCM5, and Cdc6 labeling [7]. Adult myocytes are unable to reenter the cell cycle since they express $p21^{Cip1}$, but not p53 and $p16^{INK4a}$. Old myocytes express p53 and p21 in the absence of $p16^{INK4a}$ and senescent myocytes express p53 and $p16^{INK4a}$. $p16^{INK4a}$ and p53 are markers of cellular senescence [8]. Myocyte aging and the concomitant increase in myocyte volume typically result in a severe depression in cell function and calcium metabolism.

Thus, myocytes are generated by activation and lineage commitment of CSCs–ECCs. With time, myocytes age and cellular aging is paralleled by an increase in myocyte volume, together with the expression of inhibitors of the cell cycle and markers of cellular senescence. An increase in old and senescent myocytes has detrimental effects on cardiac performance and may cause the development of a decompensated myopathy [9]. This negative impact on myocytes and organ function involves defects at the level of the regulating cell, i.e., the CSC. In fact, alterations in the stem cell compartment, i.e., the cardiac niches, lead to inadequate generation of committed progeny and inefficient replacement of senescent mechanically depressed myocytes. Apoptosis has the critical role of eliminating damaged CSCs and myocytes, and when this critical function of cell death is impaired, myocardial homeostasis is altered and cardiac performance becomes abnormal.

CSCs and their microenvironment

In self-renewing organs, cell number depends on the stem cell pool. Although stem cell antigens have been unequivocally detected in CSCs, there is no one marker capable of providing an absolute identification of stem cells *in vivo*. The localization of stem cells is as relevant as the pattern of gene expression for their identification. Stem cells are commonly stored in niches, which are located deep in the tissue for protection from damaging stimuli [10, 11]. The niche constitutes the microenvironment in which primitive cells divide, differentiate, and die. The niche provides an internal milieu in which self-renewal, differentiation, and death of stem, progenitor, and precursor cells take place. The recognition of stem cells within their niches is of crucial importance. This is why the search for the "true" stem cell removed from its microenvironment has been elusive. In fact, the true stem cell does not exist in the absence of one or more supporting cells within the niche. The stem cell is a functional unit more than a recognizable entity [12].

The identification of a stem cell niche is a difficult challenge in mammals. This search is more readily accomplished in simpler organisms such as the *Drosophila*. The majority of information about cellular components, extracellular proteins, and signaling regulatory mechanisms of niches has, in fact, been accumulated in the fruit fly [13]. The precise architectural organization of the germ cells in *Drosophila* gonads simplifies the recognition of

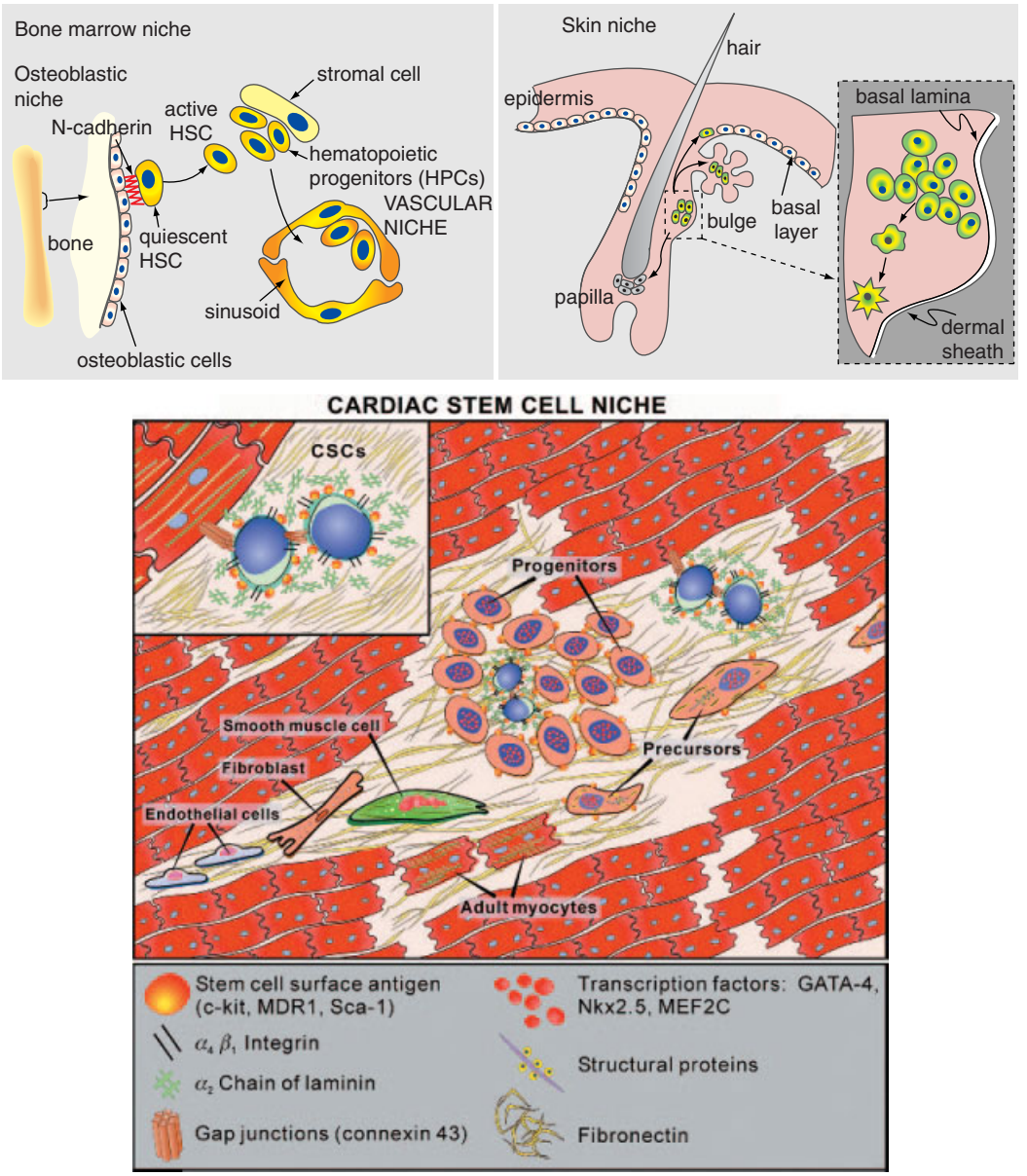


Figure 9.1 Stem cell niches. Differences in the organization of stem cells in the bone marrow, skin, and the heart.

these structures. In the fruit fly, there is a consistent reference point for the identification of the supporting cells [14]. Mammalian stem cell niches have been documented in some organs. The better defined are those in organs that possess an epithelial lining, such as the skin niches in the bulge of the hair follicles and the intestinal niches in the crypts. In these cases, the supporting or nurse cells are the dermal papilla cells and the mesenchymal cells underlying the crypt (Figure 9.1).

For the systematic identification of niches within an organ, stem cells have to be recognized, the anchoring of stem cells to the supporting cells identified and the existence of a progenitor–product relationship established [11, 13, 14]. These fundamental properties are applicable to all organs whether bone marrow, brain, or heart. This is important because the criteria employed in the study of hematopoietic stem cells cannot be transferred to neural stem cells or CSCs without caveats. For

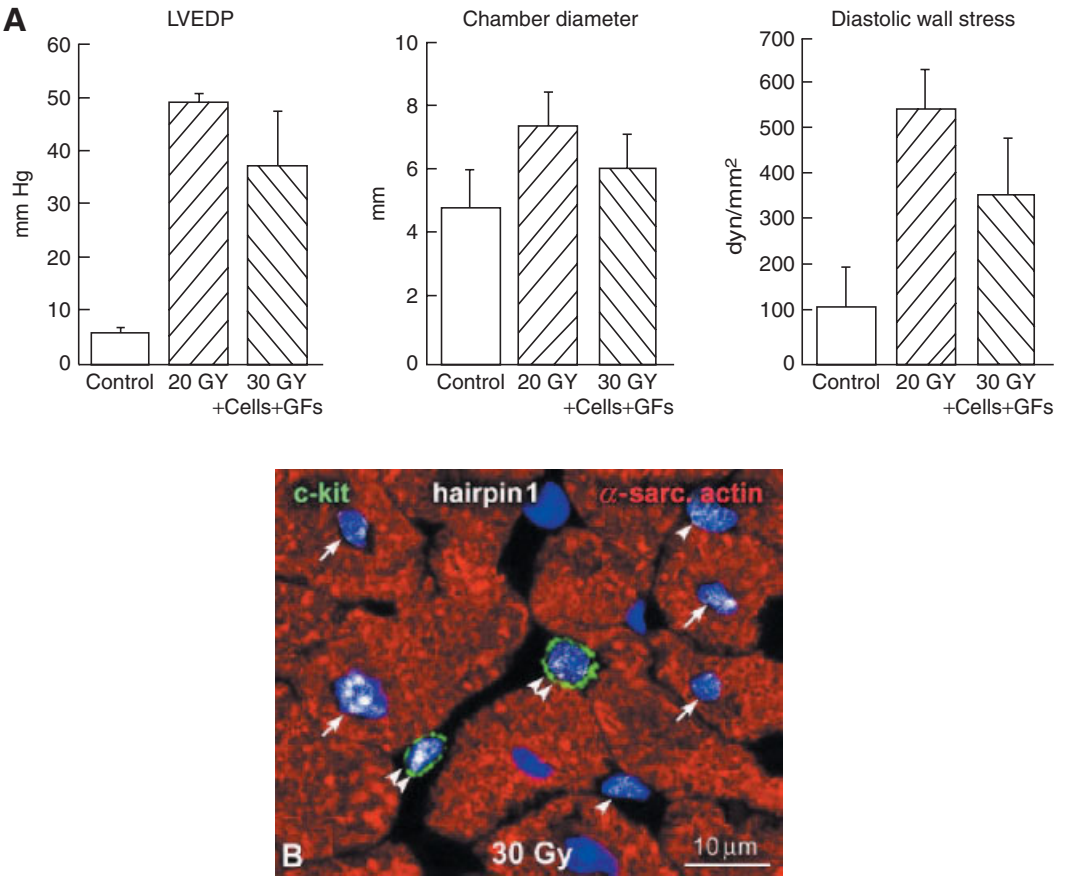


Figure 9.2 Effects of radiation on the heart. (A) Radiation of the cardiac area with 20 Gy results in an increase in left ventricular end diastolic pressure (LVEDP), chamber diameter, and diastolic wall stress. Radiation with 30 Gy leads to death acutely. If animals are exposed to 30 Gy and immediately injected with cardiac clonogenic cells and growth factors, they survive and 35 days later have very high LVEDP. However, the increases in LVEDP, chamber diameter, and diastolic wall stress are lower than in rats exposed to 20 Gy, which were not treated. (B) The confocal picture shows apoptosis (hairpin 1, white) of myocytes (α-sarcomeric actin, red, arrows), interstitial cell nuclei (arrowheads), and c-kit± cells (double arrowheads) in a rat that died 3 days after 30 Gy. Severe congestion of the lungs and liver was found.

example, the radiation protocol commonly used for lethal irradiation and bone marrow reconstitution would not be effective in the heart. In fact, the radiation dose required to reach and kill CSCs is 30 Gy. The need for 30 Gy is dictated by the structural and physical properties of the cardiac muscle [15]. This very high dose, even though restricted to the heart, results in profound alterations of the entire organ, diffuse apoptosis, and the animals die in congestive heart failure in 3–5 days. Syngeneic animals treated with growth factors together with clonogenic CSCs immediately after radiation of the cardiac area survive, but have severe ventricular dysfunction with an extremely high left ventricular end diastolic pressure (Figure 9.2). These data also demonstrate that the circulating pool of hematopoietic progenitor cells alone or in combination with those of other organs cannot replenish a depleted tissue such as the myocardium restoring physiological homeostasis. The limitations inherent in the therapeutic potential of the circulating blood should not come as a surprise. If this were not the case, spontaneous regeneration would be commonly found and myocardial infarcts, brain damage, and ischemic foci in all organs would be rapidly repaired.

Therefore, a comment has to be made concerning the viewpoint that the true CSC has to be identified and a single CSC has to be shown to possess the ability to repopulate the depleted heart before conclusions can be reached regarding the existence of CSCs [1, 16]. The heart cannot be ablated of its CSC–ECC population, and the injected single cell would have no competitive growth advantage with respect to the remaining endogenous CSCs. The belief that the bone marrow is the gold standard for any identification and characterization of stem cells [16] has to be corrected. A major effort has been made to develop the most appropriate protocol to study stem cells in solid organs where they are dispersed in the tissue, and after their identification, the critical information involves a description of the properties of the microenvironment where they reside and whether, upon activation, repair of the damaged organ can occur[4, 5].

CSC Niches

In self-renewing organs, niches constitute protected regions that contain pockets of stem cells, progenitors, and precursors located in ill-defined zones of the interstitium [13]. Recently, CSC clusters have been found in the adult heart [4, 5]. Although CSC clusters are scattered throughout the myocardium, their distribution appears to be conditioned by distinct levels of wall stress. In fact, the frequency of CSC clusters is inversely related to the hemodynamic load sustained by the anatomical regions of the heart: they accumulate in the atria and apex and are less numerous at the base and midportion of the left ventricle. The atria and apex, because of their characteristics—thin wall and low level of blood flow—do not contribute significantly to the pump function of the heart. Lin^- cells expressing a stem cell marker, c-kit, MDR1, or Sca-1, separately or in combination, have been detected, clustered together with cardiac progenitors and precursors [17]. In close proximity to Lin^- cells, myocytes or other cells in an early stage of differentiation are commonly visible. The structural organization of these sites of accumulation of poorly differentiated cells differs from that described in the bulge region of the skin [10], seminiferous tubes of the testes [18], and gut crypts [19]. In contrast, similarities in the spatial arrangement of candidate stem cells and committed cells can be found in the mouse bone marrow [20] and rat brain [21], providing elements of analogy between these organs and the heart (Fig. 1). Nurse cells in these organs have been characterized. The supporting nurse cells of CSC niches have not been identified yet. Some parameters have been evaluated in an attempt to detect the nurse cells of the cardiac niche [22].

The expression of different connexin and cadherin proteins has been detected in human atrial niches. Connexins are gap junction channel proteins that mediate passage of small molecules and signals involved in cell-to-cell communication. Also, these gap junctions anchor CSCs to a niche and interfere with their activation, commitment, and migration out of the niche [11]. Conversely, CSC depletion would occur and the entire niche would disappear. Cadherins are calcium-dependent transmembrane adhesion molecules that have a dual function; they anchor CSCs to their microenvironment and promote interaction between stem cells and between stem cells and supporting cells [22]. Preparations of freshly isolated CSCs–ECCs express connexin 43, connexin 45, N-cadherin, and E-cadherin. Moreover, these junctional and adhesion proteins are present in the plasma membrane of isolated CSCs–ECCs. Importantly, connexin 43 and 45, and N-cadherin and E-cadherin are consistently found between two CSCs, one CSC and one ECC, or two ECCs. These connexins and cadherins are also seen between CSCs–ECCs and myocytes and cardiac fibroblasts (Figure 9.3). To avoid cross-reactivity of antibodies and unspecific staining, this morphological analysis has been performed by labeling each primary antibody with a fluorochrome recognized by the confocal microscope [2].

A functional assay was introduced to characterize the role of connexins in the formation of gap junctions between CSCs–ECCs and the potential supporting cells, cardiomyocytes, and fibroblasts. Calcein dye coupling of cells was studied by two-photon microscopy following preincubation of the progenitor cells with the methyl-ester form of the dye. Because of the entry and trapping of the dye into the cells, CSCs–ECCs became green fluorescent. In addition, these cells were stained with the red fluorescent dye DiI. Fluorescent primitive cells were then plated together with cardiac cells. The presence of green fluorescence only, in the absence

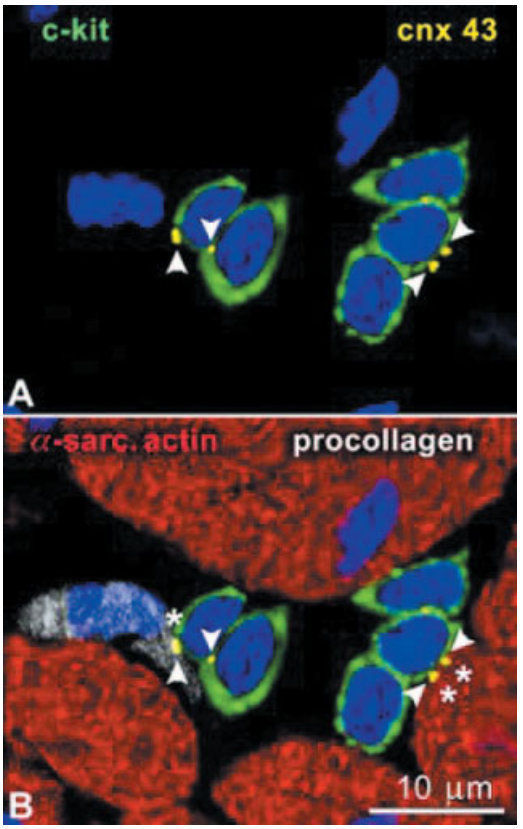


Figure 9.3 Cardiac niche. Cluster of c-kit± CSCs (A; green) showing connexin 43 on the surface of the cells (A; yellow dots, arrowheads). c-kit± CSCs are connected by connexin 43 with a myocyte (B; α-sarcomeric actin, red, asterisk) and a fibroblast (B; procollagen, white, asterisk).

of red fluorescence, indicated the transfer of calcein through gap junctions and allowed us to detect the nurse cells. Calcein translocated from CSCs–ECCs to both myocytes and fibroblasts present in the culture points to myocytes and fibroblasts as the nurse cells in the heart. Additional studies were then conducted to identify the anatomical localization of slowly cycling CSCs. On the basis of the 5-Bromo-2-deoxyuridine chasing assay, the atria were found to be the site of storage of the long-term label-retaining cells [22]. This new information, together with previous findings [5, 6, 23–25], strongly supports the notion that the heart is an organ possessing a stem cell compartment that regulates the physiological turnover of cardiac cells. Whether during development CSCs originate from hematopoietic islands in the liver or primordial structures remains to be identified and is an important question. Recent observations, however, indicate that CSCs originate from the neural crest [26].

Connexin 43 and 45 are present in the cardiac niches, but N-cadherin and E-cadherin are the most likely candidate adhesion molecules that keep the stem cells within the niches and preserve their stemness. However, an abnormal increase in the expression of N-cadherin and E-cadherin may alter the physical interaction between stem cells and their supporting cells. Enhanced expression of N-cadherin and E-cadherin in CSCs may impinge upon their locomotion out of the niches. The renewal and differentiation of CSCs may be affected, and a time-dependent increase of nonreplicating CSCs within the niche may occur. With pathologic stimuli, cardiac homeostasis may be altered and old CSCs may cluster in the niches, leading to a reduction in the number of functionally competent cells. Ultimately, the replacement of damaged myocytes in the ventricle may be impaired and organ decompensation may occur.

Similar to other self-renewing organs, such as the skin [10], the intestine [19], the bone marrow [16, 20], and the brain [21], CSCs within the niche constitute the cell compartment that constantly replenishes the myocardium with repopulating differentiated cells. The maintenance of an appropriate cell number in the heart appears to depend on the regulatory system that is operative at the level of the CSC niches. The homeostasis of a niche is dictated by a combination of several cellular processes, including proliferation, differentiation, migration, and death. The physiological integration of these cellular events is the actual determinant of the healthy state of the niches in each organ and, thereby, in the organism. This is why one of the main objectives of future investigation is the demonstration that the primary control of cardiac cell number occurs within the niches and that alterations of this mechanism lead to the abnormal phenotype typical of acquired cardiomyopathies: accumulation of old CSCs and myocytes, which together constitute ventricular dysfunction.

Niche homeostasis

Within a CSC niche, the homeostatic balance between proliferation and apoptosis has to be preserved. Whether a CSC is destined to divide or die

is only partially a cell-autonomous process. The intracellular control of cell division and death is dependent on environmental cues capable of informing cells of the needs of the tissue and the organ. When an excess of cells is formed, apoptosis occurs and when too many cells are lost, proliferation signals are generated. In this regard, the formation of organs in prenatal life is regulated by a negative feedback loop between cell multiplication and cell number [27]. When the number of cells that is physiological for that organ in that species is achieved, organ development reaches completion and cell division is repressed. Thus, the size of the organ is modulated by communications between cells and interaction of cells with the microenvironment. These dialogs enable the existing cells to compare themselves to the neighboring cells and divide or stop dividing [28]. These mechanisms of organ and tissue homeostasis have been well characterized in embryonic and fetal development, but whether they are operative in the adult heart and impaired in cardiac diseases remain to be shown. Similarly, it is unknown whether tissue regeneration in pathologic states mimics embryonic and fetal development.

However, it is intuitively apparent that this critical balance may be altered by suppression of apoptotic signals and the accumulation of unwanted cells that have lost their specialized functions. Inhibition of apoptosis during fetal life contributes to congenital diseases in the newborn [29]. In a comparable manner, excessive cell death impairs organ formation prenatally and participates in the manifestation of pathologic states after birth [30]. Cardiac failure leads to a time-dependent accumulation of old, poorly contracting myocytes and nonfunctionally competent CSCs [4, 9, 31]. Whether environmental cues that regulate embryonic and fetal growth are recapitulated in the adult heart and are lost in the presence of cardiac decompensation is poorly understood.

Early studies on the properties of stem cells in the bone marrow and intestine have led to the conviction that the number of stem cells within the niches depends on the predominance of symmetric or asymmetric division of these undifferentiated cells, in the absence of stem cell death. The possibility that stem cells may die by apoptosis was not introduced because stem cells were viewed as a unique population of ever young, immortal cells. However, this was not the case for progenitor and precursor cells. The number of these differentiating cells was thought to be regulated by cell division and cell death. More recent studies have challenged this paradigm and emphasized the importance of apoptosis not only in the control of the number of committed progenies but also in the control of stem cells in the niches and throughout the parenchyma [32]. This has been found to be true for the bone marrow [32], the intestine [33], the brain [34], and the heart [4, 9, 31]. Within a niche, the loss of a stem cell can be compensated by the replication of an adjacent stem cell that generates two self-renewing daughter cells. Alternatively, the death of a stem cell can be counteracted by the migration of a stem cell from a neighboring niche. The translocated stem cell reconstitutes the cell population of the niche with its optimal number of primitive and committed cells. The recognition of these interrelated phenomena in a solid organ such as the heart is extremely complex and, with the methodologies currently available, appears almost as an impossible task. The only protocol that can be applied to characterize the cellular events that control the life of a niche requires the removal of the existing cells followed by its repopulation with new, labeled stem cells [13]. So far, data have only been obtained in the ovary of simple organisms, such as the fruit fly [14].

Conclusions

The heart is now viewed as a self-renewing organ characterized by resident CSCs stored in niches. The niches control the physiological turnover of myocardial cells and the growth, migration, and commitment of primitive cells leaving the niches to replace old dying cells in the myocardium. The number of CSCs together with the number of ECCs is postulated to remain constant within the niches throughout life. However, changes in the cellular composition of the niches may occur in pathological conditions, leading to the accumulation of senescent, nondividing CSCs. The decrease in the number of young CSCs and the increase in the number of old CSCs within the niches may lead to alterations in myocardial homeostasis and ventricular function typically present in the decompensated heart.

Acknowledgment

This work was supported by NIH grants.

References

1 Chien KR. Stem cells: lost in translation. Nature 2004; 428:607–608.

2 Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005; 85:1373–1416.

3 Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001; 344:1750–1757.

4 Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 2006; 113:1451–1463.

5 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.

6 Pfister O, Mouquet F, Jain M, et al. CD31- but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 2005; 97:52–61.

7 Urbanek K, Quaini F, Tasca G, et al. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci USA 2003; 100:10440–10445.

8 Sherr CJ. Principles of tumor suppression. Cell 2004; 116:235–246.

9 Chimenti C, Kajstura J, Torella D, et al. Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure. Circ Res 2003; 93:604–613.

10 Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000; 287:1427–1430.

11 Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. Cell 2004; 116:769–778.

12 Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell 2001; 105:829–841.

13 Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature 2001; 414:98–104.

14 Lin H. The stem-cell niche theory: lessons from flies. Nat Rev Genet 2002; 3:931–940.

15 Ward JF. Complexity of damage produced by ionizing radiation. Cold Spring Harb Symp Quant Biol 2000; 65:377–382.

16 Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell 2004; 116:639–648.

17 Anversa P, Kajstura J, Nadal-Ginard B, Leri A. Primitive cells and tissue regeneration. Circ Res 2003; 92:579–582.

18 Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci USA 2001; 98:6186–6191.

19 Battle E, Henderson JT, Beghtel H, et al. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 2002; 111:251–263.

20 Yoshimoto M, Shinohara T, Heike T, et al. Direct visualization of transplanted hematopoietic cell reconstruction in intact mouse organs indicates the presence of a niche. Exp Hematol 2003; 31:733–740.

21 Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 2000; 425:479–494.

22 Leri A, Bearzi C, Cascapera S, et al. Symmetric and asymmetric division of cardiac stem cells in the mouse heart. Circulation 2003; 108:iv–33.

23 Oh H, Bradfute SB, Gallardo TD, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 2003; 100:12313–12318.

24 Matsuura K, Nagai T, Nishigaki N, et al. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004; 279:11384–11391.

25 Rosenblatt-Velin N, Lepore MG, Cartoni C, et al. FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. J Clin Invest 2005; 115:1724–1733.

26 Tomita Y, Matsumura K, Wakamatsu Y, et al. Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol 2005; 170:1135–1146.

27 Raff MC. Size control: the regulation of cell numbers in animal development. Cell 1996; 86:173–175.

28 Gomer RH. Not being the wrong size. Nat Rev Mol Cell Biol 2001; 2:48–54.

29 Itoh T, O'Shea C, Linn S. Impaired regulation of tumor suppressor p53 caused by mutations in the xeroderma pigmentosum DDB2 gene: mutual regulatory interactions between p48(DDB2) and p53. Mol Cell Biol 2003; 23:7540–7553.

30 Meng X, Klement JF, Leperi DA, et al. Targeted inactivation of murine laminin gamma2-chain gene recapitulates human junctional epidermolysis bullosa. J Invest Dermatol 2003; 121:720–731.

31 Urbanek K, Torella D, Sheikh F, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 2005; 102:8692–8697.

32 Domen J, Cheshier SH, Weissman IL. The role of apoptosis in the regulation of hematopoietic stem cells: overexpression of Bcl-2 increases both their number and repopulation potential. J Exp Med 2000; 191:253–264.

33 Renehan AG, Bach SP, Potten CS. The relevance of apoptosis for cellular homeostasis and tumorigenesis in the intestine. Can J Gastroenterol 2001; 15:166–176.

34 Lindsten T, Golden JA, Zong WX, et al. The proapoptotic activities of Bax and Bak limit the size of the neural stem cell pool. J Neurosci 2003; 23:11112–11119.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

10

CHAPTER 10

Activation of the local regenerative system of the heart

Antonia Germani, PhD, *Federica Limana,* PhD, *&*
Maurizio C. Capogrossi, MD

Regeneration of the adult heart can occur in newt and zebrafish, and in these lower vertebrates it involves cardiomyocyte cell cycle reentry. The adult mammalian cardiomyocytes do not have such characteristics. However, several efforts have been made to override cell cycle checkpoints to induce their proliferation. In addition, the recent identification of resident cardiac stem cells (CSCs) in several animal species [1, 2] and human beings [3, 4] suggests that the heart has intrinsic regenerative abilities. This chapter will discuss the mechanisms for cardiac regeneration in lower vertebrates, the key cell cycle mechanisms that limit adult myocardial cell proliferation, the activation of the local regenerative system of the mammalian heart in response to pathophysiologic conditions, and the possibility of using cytokines or growth factors to promote CSC proliferation and differentiation *in vivo*, without the need for *ex vivo* manipulation.

Heart regeneration in lower vertebrates

Regeneration is a complex biologic process involving the reconstitution of lost or injured tissue and organs. Among vertebrates, the newt and axolotl have remarkable regenerative abilities, which they retain during adulthood. Regenerated structures include the tail, limb, spinal cord, jaw, retina, lens, optic nerve, intestine, and heart [5]. The cellular mechanisms that control regeneration in the limb, tail, and jaw are similar and consist of the reentry of differentiated cells next to the site of injury into the cell cycle, followed by their dedifferentiation and proliferation to form tissues of regenerated structures or organs. Specifically, the first step is characterized by the formation of a wound covered by epithelial cells. This event is followed by a phase of dedifferentiation that leads to the formation, just beneath the wound epithelium, of an aggregate of undiffer entiated cells, the blastema. The final step consists of the restoration of the injured structure through the differentiation of the blastema cells into the cells and tissue of the intact organ.

Heart regeneration has been studied in the newt [6], the axolotl [7], and recently also in the adult zebrafish [8] and does not involve the formation of a morphologic equivalent of the blastema [9]. In order to study the regenerative process in the myocardium of these organisms, the heart is subjected to the removal of a large portion of the left ventricle, including the apex. Shortly after the surgical resection, the ventricular wall is sealed by the formation of a fibrin clot which, in approximately 1 month, is replaced by regenerated tissue indistinguishable from the surrounding myocardium [8, 10]. Several questions arise concerning the origin of the different cell types that form the regenerated tissue. Recent studies strongly suggest that in the adult zebrafish and axolotl models of heart regeneration, newly formed cardiomyocytes and derived from resident cardiomyocytes that reenter the cell cycle in response to injury [7–9]. It has been recently reported that the adult mammalian heart possesses resident CSCs that have the ability to proliferate and differentiate into cardiomyocytes, endothelial and

smooth muscle cells both in physiologic and pathologic conditions [1]. Whether CSCs are present in the hearts of newt, axolotl, and zebrafish and whether they contribute to heart regeneration remain open questions.

Little is known about the molecular mechanisms regulating the regenerative process in vertebrates. Recently, using mutagenesis screens, some genes involved in zebrafish heart regeneration have been identified. Msx genes encode a transcriptional repressor that prevents differentiation during development and regeneration. These genes are up-regulated in the regenerating myocardium of adult zebrafish. Also, early after resection components of the Notch pathway show an up-regulation [11]. It is noteworthy that Notch activation is a fundamental event for the proliferation/differentiation in several lineages of resident stem cells. Interestingly, these genes are not expressed during zebrafish heart development, strongly suggesting that there may be a clear distinction between the molecular mechanisms involved in development and regeneration.

Information about the regenerative processes in lower vertebrates may provide novel insights into the mechanisms that may enhance a mammal's regenerative potential. Although mammals exhibit a limited regenerative ability, they can replace tissues, such as skin, bone, peripheral nerve, and skeletal muscle. The formation of local scar tissue can dramatically inhibit most mammalian tissue regeneration, as is the case after heart injury. It has been reported that the MRL mouse, a strain with a unique capacity of regenerative wound healing, can recover with little or no scar formation after myocardial infarction of the right ventricle [12]. The hearts of these mice, when injured with a cryoprobe, appear to activate a regenerative event similar to that found in amphibians with cardiomyocyte proliferation and no fibrosis. More recently, the results of two different studies have challenged these findings, showing no differences in scar formation in MRL mice compared to C57/Bl6 controls, after both ischemia/reperfusion injury and after myocardial infarction induced by coronary artery ligation [13, 14].

Cardiomyocyte proliferation

It is commonly believed that mammalian cardiomyocytes withdraw from the cell cycle shortly after birth, and postnatal growth of the heart is largely achieved by hypertrophy rather than by hyperplasia of adult myocardial cells. Although proliferating myocytes have been detected in the heart in several physiopathologic conditions, recent experimental evidence strongly suggests that these cells derive from resident cardiac precursor cells, rather than from adult myocardial cells [15, 16]. The molecular mechanisms accounting for the irreversible cell cycle withdrawal of adult cardiomyocytes are poorly understood. However, after heart damage, adult cardiomyocytes reexpress a subset of fetal genes, suggesting that they may not be irreversibly fixed in the differentiated state. Cell cycle progression is regulated at multiple checkpoints by a complex system of proteins that coordinate the biochemical activities required for cell division. These proteins include cell cycle promoting factors such as cyclin D, A, E, and B, cyclin-dependent kinases (Cdks), cell cycle inhibitors such as Cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$, and members of the retinoblastoma (Rb) family.

The assumption that adult cardiomyocytes are nonreplicating cells is supported by the observation that positive cell cycle regulators, cyclins and Cdk, are highly expressed in the embryonic heart and down-regulated after birth, whereas negative cell cycle regulators and Cdk inhibitors are increased in the adult heart.

Considerable effort has been made to induce cardiomyocyte proliferation by manipulating the expression of oncogenes and cell cycle regulatory proteins [17]. It is noteworthy that a variety of interventions aimed at inducing cardiomyocyte proliferation have resulted in their entry into the cell cycle with the induction of DNA synthesis and chromosomal duplication in the absence of cytokinesis. However, when oncogenes or cell cycle promoters were expressed by transgenic approaches, cytokinesis was detected in adult animals, suggesting that this effect may involve alterations of the differentiation program of cardiomyocytes during fetal development. According to this hypothesis, transgenic expression of c-myc proto-oncogene in the heart enhances hyperplastic growth [18], while inducible expression of c-myc in adult cardiomyocytes fails to promote cardiomyocyte cytokinesis [19].

Viral inactivation of tumor suppressor genes may reactivate the cell cycle in cardiomyocytes. For example, targeted expression of the adenoviral protein E1A, which binds and inactivates pRb family

members, promotes G1 cell cycle exit [20]. Unfortunately, this effect is followed by increased cardiomyocyte apoptosis, probably because of activation of the tumor suppressor p53. In contrast, the expression of SV40 large T antigen, which inactivates not only pRb but also p53 and the proapoptotic protein p193, overrides G1 arrest without inducing cell death [21, 22].

Transgenic mice overexpressing cyclin D1 show elevated levels of DNA synthesis [23], while cyclin A2 overexpression [24] as well as p27 deletion result in cardiomyocyte hyperplasia [25]. The role of D type cyclins in the reactivation of cardiomyocyte proliferation has been recently addressed by comparing the effect of the transgenic expression of cyclin D1, D2, and D3 in mouse hearts [26]. All mice exhibit high rates of cardiomyocyte DNA synthesis, but only in hearts overexpressing cyclin D2 after infarction does cardiomyocyte proliferation occur, which results in reduced infarct size. The regenerative effect involves the nuclear activity of cyclin D2, which persists in the nucleus after cardiac injury; in contrast, cyclin D1 and D3 accumulate in the cytoplasm.

It is possible to enhance cardiomyocyte proliferation through interventions that modulate, at the same time, negative and positive regulators of the cell cycle. Recently it has been shown that p38 mitogen-activated protein (MAP) kinase inhibition results in cardiomyocyte proliferation *in vivo* and *in vitro* [27, 28]. p38 activation inhibits cell cycle progression via its effect on cell cycle proteins, i.e., p21, cyclin D and B. Therefore, p38 inhibition may result in cell cycle entry of cardiomyocytes. This is the first study in which cytokinesis was detected not only in cardiomyocytes derived from p38MAP kinase knock-out mice but also in wild type adult cardiomyocytes after treatment with the specific p38MAP kinase inhibitor (SB203580). Interestingly, simultaneous stimulation with fibroblast growth factor (FGF)-1 and inhibition of p38MAP kinase results in approximately 7.2% of proliferating myocytes undergoing a process that involves their transient dedifferentiation [27, 28].

Cardiac stem cell activation

CSCs are involved in the lifelong process of maintaining cardiac homeostasis and replacing, at least in part, dead cardiac cells. However, CSCs are unable to completely prevent the age-dependent decline in cardiac function and regenerate cardiac tissue after acute and chronic injury. Several mechanisms may account for the inability of CSCs to keep the heart young and healthy forever: (a) cardiac damage due either to aging or any disease process that likely involves CSCs as well as all other differentiated cells in the heart, (b) the CSC number may be too low to effectively repair extensive cardiac damage, (c) the inflammatory signals in the injured area may have negative effects on CSC proliferation and differentiation, (d) growth factors released by damaged tissue may not be sufficient to recruit stem cells to the site of injury or to provide a fully adequate stimulus for CSC proliferation and differentiation, and (e) the scar produced after myocardial injury may limit the access of CSCs to the injured area. The inhibitory effect of scar in tissue regeneration has been widely documented: injury without scarring occurs in embryos and in lower vertebrates, including urodele amphibians and zebrafish, which show a high degree of tissue regeneration.

Nevertheless, CSC involvement and activation have been shown to occur in some physiopathologic conditions. The CSC pool is enhanced acutely after infarction [4, 29]. In mice, c-kit expressing CSCs are 0.2% of the total heart cells after separation from cardiomyocytes and increase up to 1% in acutely infarcted hearts [29] (Figure 10.1). In human hearts, there are approximately 4000 CSCs per cm^3; after infarction, this number increases to 40,000 CSCs per cm^3 of tissue in the border zone and up to 20,000 CSCs per cm^3 are detected in the remote region [4]. CSC growth is also present in the myocardium of patients with chronic aortic stenosis where the increased cardiac mass results from both CSC activation and myocyte hypertrophy [30].

A different scenario is present with aging, diabetes, and heart failure [31–33]. These conditions are characterized by an increasing growth of CSCs, but apoptosis prevails on proliferation, and this imbalance reduces the number of functionally competent CSCs. In patients with cardiac aging and heart failure, the number of apoptotic c-kit^+ undifferentiated cells is high and is always associated with the expression of p16—an established marker of cellular senescence [31, 32, 34]. Also, diabetes and age-dependent effects on CSCs are attenuated in $p66^{ShcA}$ null mice [33] and in insulin growth factor-1

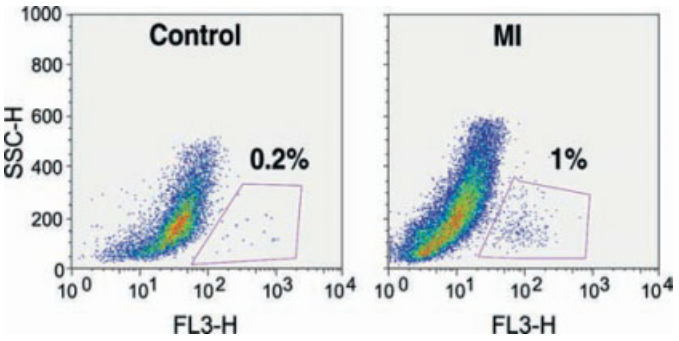


Figure 10.1 c-kit$^+$ CSCs increase in the mouse infarcted heart. Flow cytometric analysis shows c-kit$^+$ cells in noninfarcted (control) and in 24 hour postmyocardial infarction (MI) mouse heart. c-kit$^+$ cells increase from 0.2 to 1%.

(IGF-1) transgenic mice [32, 34], respectively. The p66^{ShcA} null mouse fails to express p66^{Shc} adaptor protein—a downstream target of p53 required for a p53-dependent increase of intracellular oxidants and cell death. Therefore, p66^{ShcA} null mice exhibit lower levels of intracellular reactive oxygen species and increased resistance to cell death induced by oxidative stress [35, 36]. Cardiomyopathy associated with cellular aging is delayed in IGF-1 transgenic mice where the number of functionally competent CSCs is increased. The regenerative effect of IGF-1 is also documented in skeletal muscle, where IGF-1 overexpression increases skeletal muscle stem cell activity and prevents age-related loss of muscle size and strength [37]. Therefore, identification of factors that enhance both the activation and/or the survival of CSCs may limit the detrimental effects of both diabetes and age-dependent declines in cardiac function (see Chapter 16 & 17).

The mechanisms involved in CSC activation in response to injury are still largely unknown. After myocardial infarction a plethora of growth factors and cytokines are produced, and it is difficult to define the effect of each factor on CSCs because of pleiotropic, redundant, and time-dependent actions. The early phase of myocardial infarction includes the necrotic and apoptotic death of cardiomyocytes and the activation of the inflammatory response. Dead cells as well as inflammatory cells are sources of cytokines and growth factors that are important for the cardiac remodeling process. Some of these factors are proangiogenic, e.g., vascular endothelial growth factor (VEGF), FGF-2 and FGF-1, and hepatocyte growth factor (HGF). Other factors have been involved in the mobilization of bone-marrow-derived stem cells (BMSC) after infarction and include serum-derived factor-1 (SDF-1) and erythropoietin (EPO) [38]. It is still unclear whether these factors, which are known to modulate some BMSC functions, have effects on CSCs as well.

Interestingly, some growth factors including VEGF, FGF-1, FGF-2, EPO, and HGF have been previously delivered either as recombinant protein or by gene therapy as an approach for providing cardiac protection and for increasing the blood supply in animal models of myocardial ischemia, infarction, and heart failure [39]. With the identification of CSCs, the results reported in these studies should be reevaluated in order to determine whether there was any effect on CSCs.

FGF-2 expression is up-regulated after cardiac injury and it has been implicated in cell proliferation survival and differentiation [40]. Moreover, FGF-2 knock-out (FGF$^{-/-}$) mice show absence of cardiac remodeling in response to pressure overload [41]. FGF-2 and its receptor (FGFR1) are expressed in a subset of CSCs, and their interaction is required for cardiogenic differentiation *in vitro* and *in vivo*. Indeed, CSCs derived from FGF-2$^{-/-}$ mice, when injected intravenously, either in wild type or in FGF-2$^{-/-}$ mice, home to the heart but differentiate into cardiomyocytes only when the recipient animals produce FGF-2 [42]. Other members of the FGF family may have similar properties. FGF-5 delivery to a swine model of hibernating myocardium improves blood flow and induces cell cycle reentry of small cardiomyocytes; it is possible that these small myocardial cells derive from CSCs [43, 44].

Another factor of interest is Sonic Hedgehog (Shh). This is a secreted glycoprotein involved in developmental processes, including heart morphogenesis, as shown by the presence of a heart-looping defect in *Shh*–/– mice [45]. Further, Shh is an

angiogenic factor that up-regulates VEGF and angiopoietins-1 and -2 in interstitial mesenchymal cells [46]. Myocardial gene therapy with Shh enhances neovascularization and reduces fibrosis as well as apoptosis through a mechanism that involves BMSC recruitment in the heart [45]. However, whether or not Shh has an effect on CSCs has not been investigated.

The possibility of promoting cardiac tissue regeneration through growth-factor-mediated CSC activation has been recently addressed. CSCs ex press receptors for a variety of growth factors, cytokines, and chemokines; these include the HGF receptor c-Met, IGF-1R, VEGF receptor, stem cell factor (SCF) receptor, epidermal growth factor (EGF) receptor, FGF-R1, SDF-1 receptor CXCR4 [47], and the receptor for advanced glycation end products (RAGE) which is also activated by the cytokine high mobility group box 1 (HMGB1) [29]. Therefore, growth factor treatment may modulate CSC function.

Recently, IGF-1 and HGF have been used in combination in order to stimulate the migration of resident CSCs to the infarcted area of the heart and promote their proliferation and survival. These experiments were performed in mice [48] and dogs [49], in which CSCs were isolated and characterized. IGF-1 plays an important role in the homeostasis of the CSC compartment. As previously reported, overexpression of the IGF-1 transgene selectively in the heart promotes cardiomyocyte formation and reduces myocyte death after infarction [32, 34]. In the aging myocardium, the IGF-1/IGF-1R system increases CSC proliferation and reduces CSC apoptosis. These two processes lead to a delay in the senescence of CSCs, increasing the number of these cells that are functionally competent.

HGF may promote cell migration in various organs, including the brain, and, to a lesser extent, cell growth and survival. Cell locomotion is activated by the expression and activation of matrix metalloproteases that digest the extracellular matrix.

In vitro studies show that HGF is a powerful CSC chemoattractant, more effective than FGF-2, granulocyte-macrophage colony stimulating factor (GM-CSF), EGF, VEGF, and SCF. On the other hand, IGF-1 shows little effect on CSC migration but has a predominant role in promoting their proliferation and survival. In animal studies, the administration of HGF and IGF-1 in infarcted hearts induced CSC migration in the injured region where they differentiated into cardiomyocytes and coronary vessels. In mice, at 16 days the volume of newly formed myocytes ranged from 600 to 7200 μm^3 and at 4 months it ranged from 700 to 20,000 μm^3. In dogs, at 28 days the size distribution of regenerated myocytes ranged between 400 and 17,000 μm^3. Since adult murine and canine myocytes have an average volume of 20,000 and 25,000 μm^3, respectively, myocardial regeneration in both species was characterized by the formation of immature cardiac cells that resemble a fetal–neonatal phenotype. However, in both animal models, in response to treatment with HGF and IGF-1, there was echocardiographic evidence of contraction in the infarcted region, and hemodynamic studies documented an improvement in cardiac function. These results were confirmed by another study performed in rats, in which the regenerative response of resident CSCs was documented after the local injections of HGF and IGF-1 in chronic infarcts [50].

In addition to the combination of HGF and IGF-1, it has also been shown that the cytokine HMGB1 can modulate CSC function. HMGB1 is a nonhistone nuclear protein constitutively expressed in the nucleus of quiescent cells where it binds the AT sequence in the minor groove of DNA and, via this mechanism, is involved in the regulation of gene transcription and in stabilizing nucleosome formation [51]. Recently, a novel role for HMGB1 as a cytokine has been identified. HMGB1 is a potent mediator of inflammation secreted by monocyte-macrophage in response to proinflammatory cytokines and released from necrotic cells. Moreover, when added to endothelial cells, HMGB1 elicits proinflammatory responses by increasing the expression of vascular adhesion molecules as well as the secretion of cytokines and chemokines. The effects of HMGB1 are mediated by its binding to RAGE—a multiligand receptor of the immunoglobulin superfamily [52]. Extracellular functions of HMGB1 are not limited to the inflammatory response. HMGB1 is also a strong chemoattractant for vessel-associated stem cells (mesoangioblasts) [53] and induces proliferation and differentiation of CSCs [29]. The last effect has been demonstrated in a mouse model of myocardial infarction where HMGB1 administration enhanced CSC proliferation in the heart and resulted in the formation of a band of regenerated myocardium constituted by

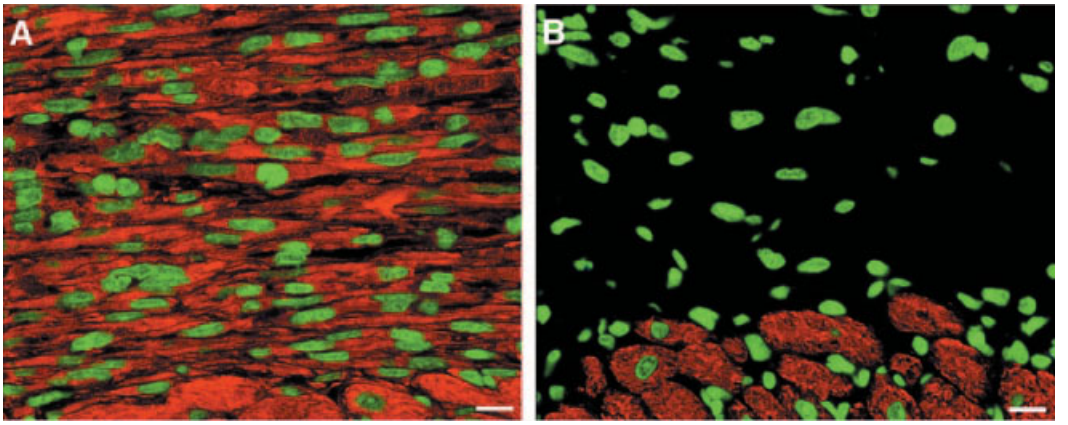


Figure 10.2 HMGB1 promotes cardiac tissue regeneration. (A) Infarcted HMGB1-treated hearts: newly formed myocytes are detected and express α-sarcomeric actin (red fluorescence). (B) Infarcted heart treated with a control protein: only surviving endocardial cells are present and express α-sarcomeric actin (green false color, propidium iodide in nuclei). (Adapted, with permission, from [29].)

newly formed cells expressing cardiac markers (Figure 10.2), i.e., the transcriptional factor MEF2C, α-sarcomeric actin, and connexin 43. These cells were smaller than adult myocardial cells, and 1 week after myocardial infarction and HMGB1 treatment, their volume ranged between 300 and 600 μm^3. Left ventricular function evaluated by echocardiography and hemodynamic measurements was significantly enhanced compared to control in the 4 weeks after coronary artery ligation. It is noteworthy that there was no change in c-kit$^+$ stem cell number in the systemic circulation nor was there enhanced c-kit$^+$ cell homing in the infarcted heart. Therefore, the ability of HMGB1 to induce regeneration of the infarcted left ventricle can be attributed to enhanced CSC proliferation and differentiation.

Other growth factors may have a similar effect on CSCs. SDF-1 has an important role in BMSC mobilization after injury. It has been shown that SDF-1 delivery in a rat model of ischemic cardiomyopathy enhanced BMSCs recruitment and new blood vessel formation in the damaged tissue [54]. Similar to BMSCs, CSCs express the SDF-1 receptor CXCR4 and migrate *in vitro* in the presence of SDF-1 through a mechanism that involves the phosphorylation of src family of tyrosine kinases [55]. Therefore, SDF-1 may be considered a mobilization factor not only for BMSCs but also for CSCs. However, it is still unknown whether SDF-1 induces CSC proliferation and differentiation.

Conclusions

At present, CSCs appear to be key players in the local cardiac regenerative system. They are activated in response to a variety of noxious stimuli and provide a potential compensatory mechanism to preserve cardiac function following damage. Further, CSCs can also be activated by exogenously administered cytokines or growth factors, and the potential therapeutic value of these interventions remains to be established. Future studies will help to establish whether, in similar fashion to what occurs in the hearts of some lower vertebrates, adult human myocardial cells can be induced to reenter the cell cycle and proliferate in order to provide an additional mechanism to repair the damaged heart.

Acknowledgments

We are thankful to Fabio Martelli for comments and critical reading of the manuscript.

References

1 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.

2 Hierlihy AM, Seale P, Lobe CG, et al. The post-natal heart contains a myocardial stem cell population. FEBS Lett 2002; 530:239–243.

3 Messina E, De Angelis L, Frati G, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004; 95:911–921.

4 Urbanek K, Torella D, Sheikh F, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 2005; 102:8692–8697.

5 Odelberg SJ. Cellular plasticity in vertebrate regeneration. Anat Rec B New Anat 2005; 287:25–35.

6 Oberpriller JO, Oberpriller JC. Response of the adult newt ventricle to injury. J Exp Zool 1974; 187:249–253.

7 Flink IL. Cell cycle reentry of ventricular and atrial car diomyocytes and cells within the epicardium following amputation of the ventricular apex in the axolotl, Am blystoma mexicanum: confocal microscopic immunoflu orescent image analysis of bromodeoxyuridine-labeled nuclei. Anat Embryol (Berl) 2002; 205:235–244.

8 Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science 2002; 298:2188–2190.

9 Raya A, Consiglio A, Kawakami Y, et al. The zebrafish as a model of heart regeneration. Cloning Stem Cells 2004; 6:345–351.

10 Keating MT. Genetic approaches to disease and regeneration. Philos Trans R Soc Lond B Biol Sci 2004; 359:795–798.

11 Raya A, Koth CM, Buscher D, et al. Activation of Notch signaling pathway precedes heart regeneration in zebrafish. Proc Natl Acad Sci USA 2003; 100(suppl 1): 11889–11895.

12 Heber-Katz E, Leferovich J, Bedelbaeva K, et al. The scarless heart and the MRL mouse. Philos Trans R Soc Lond B Biol Sci 2004; 359:785–793.

13 Oh YS, Thomson LE, Fishbein MC, et al. Scar formation after ischemic myocardial injury in MRL mice. Cardiovasc Pathol 2004; 13, 203–206.

14 Abdullah I, Lepore JJ, Epstein JA, et al. MRL mice fail to heal the heart in response to ischemia-reperfusion injury. Wound Repair Regen 2005; 13:205–208.

15 Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. N Engl J Med 2002; 346:5–15.

16 Urbanek K, Quaini F, Tasca G, et al. From the cover: intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci USA 2003; 100:10440–10445.

17 Field LJ. Modulation of the cardiomyocyte cell cycle in genetically altered animals. Ann N Y Acad Sci 2004; 1015:160–170.

18 Jackson T, Allard MF, Sreenan CM, et al. The c-myc proto-oncogene regulates cardiac development in transgenic mice. Mol Cell Biol 1990; 10:3709–3716.

19 Xiao G, Mao S, Baumgarten G, et al. Inducible activation of c-Myc in adult myocardium in vivo provokes cardiac myocyte hypertrophy and reactivation of DNA synthesis. Circ Res 2001; 89:1122–1129.

20 Kirshenbaum LA, Abdellatif M, Chakraborty S, Schneider MD. Human E2F-1 reactivates cell cycle progression in ventricular myocytes and represses cardiac gene transcription. Dev Biol 1996; 179:402–411.

21 Pasumarthi KB, Tsai SC, Field LJ. Coexpression of mutant p53 and p193 renders embryonic stem cell-derived cardiomyocytes responsive to the growth-promoting activities of adenoviral E1A. Circ Res 2001; 88:1004–1011.

22 Nakajima H, Nakajima HO, Tsai SC, Field LJ. Expression of mutant p193 and p53 permits cardiomyocyte cell cycle reentry after myocardial infarction in transgenic mice. Circ Res 2004; 94:1606–1614.

23 Soonpaa MH, Koh GY, Pajak L, et al. Cyclin D1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. J Clin Invest 1997; 99:2644–2654.

24 Chaudhry HW, Dashoush NH, Tang H, et al. Cyclin A2 mediates cardiomyocyte mitosis in the postmitotic myocardium. J Biol Chem 2004; 279:35858–35866.

25 Poolman RA, Li JM, Durand B, Brooks G. Altered expression of cell cycle proteins and prolonged duration of cardiac myocyte hyperplasia in p27KIP1 knockout mice. Circ Res 1999; 85:117–127.

26 Pasumarthi KB, Nakajima H, Nakajima HO, et al. Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. Circ Res 2005; 96:110–118.

27 Engel FB, Schebesta M, Duong MT, LuG, Ren S, Madwed JB, Jiang H, Wang Y, Keating MT p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. Genes Dve. 2005;19(10):1175–87.

28 Engel FB, Hseih PC, Lee RT, Keating MT: FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. Pro Natl Acad Sci USA. 2006; 103(42):15546–51.

29 Limana F, Germani A, Zacheo A, et al. Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac c-kit+ cell proliferation and differentiation. Circ Res 2005; 97:e73–e83.

30 Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy Proc Natl Acad Sci USA. 2003; 100(18): 10440–10445.

31 Chimenti C, Kajstura J, Torella D, et al. Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure. Circ Res 2003; 93:604–613.

32 Torella D, Rota M, Nurzynska D, et al. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 2004; 94:514–524.

33 Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME, Esposito G, Vitale S, Urbanek K, Casarsa G, Giorgio M, Luscher TF, Pelicci PG, Anversa P, Leri A, Kajstura J. Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. Circ Res 2006; 99(1):42–52.

34 Capogrossi MC. Cardiac stem cells fail with aging: a new mechanism for the age-dependent decline in cardiac function. Circ Res 2004; 94:411–413.

35 Migliaccio E, Giorgio M, Mele S, et al. The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 1999; 402:309–313.

36 Zaccagnini G, Gaetano C, Della Pietra L, et al. Activation of telomerase through VEGF signaling triggers angiogenesis in response to hindlimb ischemia. J Biol Chem 2005; 280:14790–14798.

37 Musaro A, McCullagh K, Paul A, et al. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 2001; 27:195–200.

38 Vandervelde S, van Luyn MJ, Tio RA, Harmsen MC. Signaling factors in stem cell-mediated repair of infarcted myocardium. J Mol Cell Cardiol 2005; 39:363–376.

39 Markkanen JE, Rissanen TT, Kivela A, Yla-Herttuala S. Growth factor-induced therapeutic angiogenesis and arteriogenesis in the heart–gene therapy. Cardiovasc Res 2005; 65:656–664.

40 Detillieux KA, Sheikh F, Kardami E, Cattini PA. Biological activities of fibroblast growth factor-2 in the adult myocardium. Cardiovasc Res 2003; 57:8–19.

41 Pellieux C, Foletti A, Peduto G, et al. Dilated cardiomyopathy and impaired cardiac hypertrophic response to angiotensin II in mice lacking FGF-2. J Clin Invest 2001; 108, 1843–1851.

42 Rosenblatt-Velin N, Lepore MG, Cartoni C, Beermann F, Pedrazzini T. FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. J Clin Invest 2005; 115:1724–1733.

43 Giordano FJ, Ping P, McKirnan MD, et al. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nat Med 1996; 2:534–539.

44 Suzuki G, Lee TC, Fallavollita JA, Canty JM, Jr. Adenoviral gene transfer of FGF-5 to hibernating myocardium improves function and stimulates myocytes to hypertrophy and reenter the cell cycle. Circ Res 2005; 96:767–775.

45 Kusano KF, Pola R, Murayama T, et al. Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. Nat Med 2005; 11:1197–1204.

46 Pola R, Ling LE, Silver M, et al. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nat Med 2001; 7:706–711.

47 Linke A, Muller P, Nurzynska D, et al.: Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci USA. 2005; 102(25): 8966–71.

48 Urbanek K, Rota M, Cascapera S, et al. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res 2005; 97:663–673.

49 Linke A, Muller P, Nurzynska D, et al. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci USA 2005; 102:8966–8971.

50 Kajstura J, Rota M, Chimenti S, et al. Local activation or implantation of cardiac progenitor cells rescues scarred infarcted myocardium improving cardiac function. (abst) Circ Res 2005; 97:1200.

51 Bianchi ME, Beltrame M. Flexing DNA: HMG-box proteins and their partners. Am J Hum Genet 1998; 63:1573–1577.

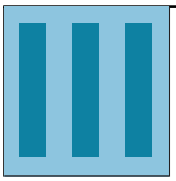
52 Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005; 5:331–342.

53 Palumbo R, Sampaolesi M, De Marchis F, et al. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol 2004; 164:441–449.

54 Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003; 362:697–703

55 Tang L, Qian K, Shen L, Phillips M. Critical role of SDF1-alpha-mediated Src signal pathway in the migration of adult cardiac stem cells [Abstract]. Circulation 2005; 112: ii–150.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman



PART III

Progenitor cells and the diseased heart

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

11

CHAPTER 11

Embryonic stem cells and myocardial regeneration

Ahmad Y. Sheikh, MD, *Phillip C. Yang,* MD, *Joseph C. Wu,* MD, PhD, *& Robert C. Robbins,* MD

Introduction

The ideal therapeutic cell candidate for myocardial therapy should have the capacity to restore myocardial function, prevent remodeling, induce native repair, and evade elimination by the host. Mechanistic pathways by which transplanted cells might achieve these effects are numerous, including the ability to self-renew, the potential for multilineage differentiation (e.g., myocardial cells, blood vessels, cardioneuronal cells), the modulation of paracrine signaling pathways, and the suppression of local host-immune responses. Because embryonic stem (ES) cells possess these aforementioned characteristics, they have gained great interest as a potential ideal therapeutic cell candidate in recent years. Although data from the benchtop are encouraging, there remain large gaps between present observations and successful, safe application of these cells in humans. This chapter will review the current status of ES cell biology, application in animal models of cardiac disease, imaging of cell therapy, and touch upon the future directions of the field (see Chap 3).

Definition

ES cells can be defined as totipotent, self-renewing cells derived from the early mammalian embryo that are capable of unlimited proliferation *in vitro* [1, 2]. Furthermore, ES cells maintain the ability to differentiate into all three embryonic germ layers even after prolonged culture [3]. To this end, investigators have demonstrated differentiation into specific cell types such as neuronal, cartilage, hepatocyte, islet, and muscle cells *in vitro*. With regards to cardiac differentiation, Kehat and colleagues demonstrated that human ES-cell-derived beating myocytes were structurally and functionally identical to early stage cardiomyocytes [4]. This phenomenon has been duplicated by several other investigators and highlights the potential of ES cells to act as true regenerative cellular agents for the heart [5–8].

The source of ES cells remains the early embryo at the blastocyst stage. Following fertilization, the mammalian zygote proceeds through the morula stage to form the blastocyst. This structure consists of a hollow sphere of cells that contains a solid inner cluster of cells. The outer layer gives rise to trophoblasts which will eventually form the placenta and other supporting structures for the developing embryo, while the inner cell mass (embryoblasts) will develop and differentiate into the various tissues of the adult body. Harvested at this stage, embryoblasts can be cultured *in vitro* and proliferate indefinitely with the potential to differentiate into all embryonic germ layers and are hence dubbed ES cells.

History of ES cell development

The first ES cell lineages were generated in the early eighties from mouse blastocysts by immunosurgical removal of the trophoblast layer followed by culturing of the inner cell mass on feeder layers of cells [9–11]. The resulting cultures produced colonies that were mechanically isolated and passaged to

establish various murine ES cell lines. By 1998, Thomson and colleagues had applied similar techniques to isolate and establish various lines of human ES cells [2]. Over the last few years, numerous laboratories have followed suit and described several other human ES cell lines [12–14].

Currently, over 400 different human ES lines exist worldwide, but only 179 of these have actually been characterized in at least partial detail and reported in scientific peer-reviewed journals [14]. In an effort to organize and facilitate human ES cell research in the United States, the National Institutes of Health created the Embryonic Stem Cell Registry (escr.nih.gov), which lists all human ES cell lines eligible for US federal funding. Presently, 71 different human ES cell lines are listed with the Registry, and only 22 of these are currently available to researchers.

Isolating and culturing ES cells

The science of procurement, cultivation, and care of ES cells has evolved into an ever-changing field unto itself. A detailed discussion of ES cell benchtop techniques is beyond the scope of this chapter; however, a few salient points regarding the cultivation of these cells will be discussed. In addition, since the majority of published and ongoing studies have focused on the biology of murine and human ES cells, a targeted comparison of these two moieties will also be presented.

Early experience with culturing ES cells demonstrated the importance of "feeder layers" to maintain pluripotency of the cells. Typically, these feeder layers consist of mitotically inactivated mouse embryonic fibroblasts (MEF), upon which ES cells are cultured directly. A number of factors produced by cells comprising the feeder layers are thought to help maintain ES cell pluripotency in culture. As reported by Xu et al., human ES cells maintained the capacity to proliferate in undifferentiated state if grown on Matrigel or laminin-containing medium conditioned by MEF [15].

Further investigations revealed various specific molecules that inhibit ES cell differentiation *in vitro*. Leukemia inhibitory factor (LIF) is one such protein that was first described by Pease and colleagues to inhibit mouse ES cell differentiation in the absence of feeder cells [16]. Interestingly, LIF does not prevent differentiation of human ES cells grown in a similar, feeder-free state [2, 12]. Such findings highlight differences between human and murine ES cells, as will be discussed later in this section. Since the discovery of LIF-based differentiation inhibitors, several other factors have been successfully used (either independently or in concert with LIF) to maintain undifferentiated ESC cultures. These include, but are not limited to, activin A [17], thrombopoetin [18], oncostatin M [19], and basic fibroblast growth factor [20–22].

The study of mouse ES cells provided enormous insight into the basics of cell biology and paved the way for isolation of primate and human ES cells. However, as murine and human ES cells are being studied further and compared, it is becoming increasingly clear that these two classes of cells differ from one another in several aspects [23]. For example, unlike mouse ES cells, human ES cells are able to differentiate into trophoblast-like cells [2, 24]. In addition, human ES cells do not appear to require LIF for propagation and maintenance in an undifferentiated state. This phenomenon may be attributed to the lack of LIF-receptor expression by human ES cells and the prominence of the receptor on mouse ES cells, as described by Rao's group [23]. In the aforementioned study, the investigators compared the expression of nearly 400 genes in a common mouse ES cell line (D3) versus an easily available human ES cell line (H1) to reveal interesting similarities and differences between the two. One of the more striking findings was the identification of an immunoreactive cell subpopulation interspersed within the undifferentiated human ES cells, whereas no such population was identified in the mouse ES cell culture. Such findings may have great implications for conclusions drawn regarding basic ES cell biology (in this case, the immunogenicity of ES cells). This only underscores the importance of careful characterization and comparison of both murine and human cell lines to effectively understand stem cell biology and safely take steps toward translation of such work into therapeutic intervention.

If human ES-cell-based transplantation therapy is to become a reality, large-scale production of cells must be achieved while minimizing the risk of pathogenic cross-contamination from animal and/or human tissue derived support systems. Hence, as upscaling technology has advanced, so too has the ability to maintain human ES cell

cultures in systems free of xenogenic and human support elements. Successful human-only preparations that allow for prolonged and undifferentiated ES cell growth have been described by several investigators. In such systems, the traditional murine MEF layer may be replaced by human ES-cell-derived fibroblast-like cells [25], combinations of adult-derived human fibroblasts and fetal cells [26], placental-derived cells [27, 28], human foreskin-derived feeders [29], or human adult marrow cells [30]. In addition to being completely human-tissue based, many of the above-described preparations also avoid the use of any serum, further reducing the risk of pathogen contamination. Most recently, several investigators have described yet another technological advance in human ES cell cultivation, whereby the feeder layer is eliminated, thus establishing true serum- and feeder-free cultures. For example, Rosler et al. described long-term (>1 year) maintenance of undifferentiated, pluripotent human ES cells in the absence of a feeder layer [31]. Similarly, Amit et al. as well as Lanza's group have published methodologies for maintaining human ES cells in the absence of both feeder cells and any serum-based products [32, 33].

Differentiation of ES cells into cardiomyocytes

Ironically, one of the remarkable qualities of ES cells, namely pluripotency, may prove to be a major obstacle to successful application for therapy. ES cell transplantation experiments in rodents have already demonstrated that undifferentiated ES cells will proliferate unchecked in the host to form teratomas [1, 34]. One strategy to combat this potential complication has revolved around inducing ES cells to first differentiate into cardiomyocytes *in vitro*. For the ideal implementation of this approach, ES cells would be grown in large numbers and coached to adopt developmental fates of cardiomyocytes. The resultant populations of cardiac progenitor cells would then be purified, ensuring no undifferentiated ES cells persisted, and then be prepared for transplantation into the host myocardium. Ideally, a redundant "check" system would be in place such that any cellular misbehavior posttransplant could also be controlled. Such a refined system is still more science fiction than fact. However, there have been many recent advances in the areas of selective differentiation of ES cells into cardiomyocytes and the subsequent purification of such populations.

In the absence of inhibitory factors, ES cells can spontaneously differentiate *in vitro* to form various types of tissues (e.g., bone, cartilage, muscle). It was observed early on that, when permitted to aggregate in culture, ES cells would form small embryo-like collections of cells, dubbed "embryoid bodies" [35]. Further refinement of culturing techniques resulted in the establishment of "hanging drop" preparations, allowing for growth of a single embryoid body in a small drop of medium suspended from the top of a culture dish [36]. As the embryoid body matures, cells can give rise to early embryonic lineages, including clusters of beating cells that contain cardiomyocytes. Unfortunately, the rate of spontaneous differentiation of embryoid bodies into cardiomyocytes is exceedingly low and leaves much room for improvement. As demonstrated by Kehat et al., only 8% of embryoid bodies grown in suspension undergo spontaneous differentiation into beating clusters and only approximately 30% of the cells contained in these clusters are actual cardiomyocytes [4]. A number of techniques have since been described that have improved the efficiency of cardiomyogenic differentiation *in vitro* and will be briefly discussed.

Strategies to induce cardiac-specific differentiation of ES cells largely rely upon modulation of culturing conditions. Included in this approach are coculturing with different cell types, alterations in serum content of the medium, introduction of endogenous growth factors, addition of various bioorganic molecules, and biomechanical manipulation of the culturing environment. Mummery et al. first reported the use of coculture of murine-derived visceral-endoderm-like cells with human ES cells in 2003 [8]. They observed that the presence of the visceral-endoderm-like cells promoted a high rate of differentiation into cardiomyocytes. Passier and colleagues later augmented this effect by eliminating serum from the coculture and supplementing with ascorbic acid [37]. Together, these manipulations resulted in a near 25-fold increase in the number of beating areas per cell preparation.

Evaluation of various growth factors and proteins has also revealed a diverse array of agents capable of inducing cardiomyogenesis from ES cells. As a collective group of peptides and secreted factors, fibroblast-derived extracellular matrix

(cardiogel) was shown, by Ashtiani's group, to promote early maturation of mouse ES-cell-derived cardiomyocytes in culture [38]. Studies evaluating specific growth factors in murine ES cell systems have revealed basic fibroblast growth factor (type 1 and 2) [39, 40], sphingosine-1-phosphate [41], platelet-derived growth factor-BB [41], transforming growth factor-β (TGF-β) (type 1 and 2) [42, 43], and bone morphogenic protein-2 [40] to promote cardiomyogenesis. Finally, hormones such as erythropoietin [44] and oxytocin [45] have also been shown to modulate early myocardial cell differentiation of mouse ES cells *in vitro*.

In addition to growth factor and hormones, several bioorganic and small molecules have been described to have procardiogenic differentiation effects upon cultured ES cells. Examples germane to mouse ES cells include dynorphin B (an endogenous opiod) [46], ascorbic acid [47], nitric oxide [48], and retinoic acid [49–51]. It is noteworthy that 5-aza-2′-deoxycytidine has been described, by Xu et al., to promote cardiomyocyte differentiation of human ES cells in culture [6].

Biomechanical modulations of the ES cell culturing environment that appear to induce an enhancement of myocardial differentiation include the introduction of laminar shear stress as conducted by Illi et al. [52] Other approaches have employed the use of electrical field modulations [53] and rotating suspension techniques [51, 54]. The latter technique of rotating suspension-based culture has proven particularly intriguing, given its ability to produce high numbers of differentiated cardiomyocytes.

Once a population of ES cells is successfully induced to adopt a cardiac fate, it becomes yet another challenge to isolate and further purify such subpopulations while avoiding contamination by pluripotent ES cells. Presently, methodologies for isolation and purification of ES-cell-derived cardiomyocytes fall into two broad categories: mechanical and genetic techniques. Mechanical isolation may be as simple as manual extraction of beating cardiomyocytes off a culture plate followed by clonal expansion. However, this is clearly not a technique suited for large-scale, high throughput culturing and isolation of cells that would be required for clinical applications. Other mechanical separation/purification methods include Percoll density gradient-based isolation as described by Xu et al. [6] Although this technique improves the throughput of isolation compared to manual excision, it still does not yield a pure population of cardiomyocytes, as the reported purity of cardiomyocytes isolated by Percoll was only 70% [6].

An alternative isolation and enrichment strategy elegantly combines the use of genetic engineering and molecular biology techniques to achieve purer populations of cardiomyocytes on a large-scale. With this approach, a fusion gene consisting of both a promoter and "selection" gene are introduced into the ES cells in culture. The promoter should ideally drive a gene that is expressed once the ES cells begin to differentiate down the cardiomyocyte pathway. The selection gene might encode for any number of proteins that could aid in the rapid and specific isolation of the differentiated cell. The choice of selection gene will depend on the methodologies preferred and available to the investigator. Klug et al. described the use of this method to obtain mouse ES-cell-derived cardiomyocyte populations that were 99% pure [55]. For their system, Klug's group used a fusion gene consisting of alpha-cardiac myosin heavy chain promoter driving expression of aminoglycoside phosphotransferase, which is an enzyme that confers resistance to the cytotoxic agent geneticin (G418). Once the transgenic mouse ES cells began to differentiate, the cardiac-specific promoter was "turned on" and thus the differentiating cells began to express aminoglycoside phosphotransferase, allowing them to survive treatment of the culture system with G418. The resultant, surviving cells were an exceedingly pure population of cardiomyocytes. Muller et al. later described a similar technique whereby mouse ES cells were transfected with a fusion gene consisting of a ventricular-specific promoter (2.1-kb myosin light chain-2v) linked to enhanced green fluorescent protein [56]. In this case, murine ES cells differentiating into cardiomyocytes expressed enhanced green fluorescent protein, which enabled the fluorescent-activated cell sorting based collection of a 97% pure cardiomyocyte population. Finally, genetic enrichment techniques have been combined with biomechanical culturing modulations to achieve large numbers of high-purity ES-cell-derived cardiomyocytes.

Specifically, Zweigerdt and colleagues combined the G418 antibiotic-driven resistance method, described above, with a rotating suspension culture

system [54]. Using this culturing system, the group produced a 99% pure population of cardiomyocytes at a concentration of 2.1×10^5 cells/mL, starting with only $2.5–8 \times 10^6$ differentiating cells 20 days prior. This study also illustrates quite nicely the synergy that can be achieved between the various methodologies discussed thus far. Indeed, there is probably no single methodology or technique that will yield the goal of high-volume, high-purity cell populations suitable for clinical use. Rather, such an objective will likely only be achieved by cross-fertilization of several technologies across various disciplines. Finally, although genetic manipulation of ES cells affords high-throughput isolation and purification of cardiomyocytes, there is much need in the way of validation and assessment of safety when translating such techniques to human ES cells intended for therapeutic use.

While the scientific community has made considerable strides in cultivating ES cells and promoting their differentiation down cardiac-specific pathways, the basic understanding of mechanisms regulating such differentiation remains poor. There are numerous pathways that have been implicated as key regulators of cardiac differentiation, and there is likely much crosstalk and overlap between them. Certainly, the signaling pathways downstream of the receptors for growth factors empirically observed to promote cardiomyocyte differentiation are of importance. Indeed, this has already been demonstrated for retinoid X receptor signaling [50] as well as for pathways downstream of the TGF-β and bone morphogenic protein-2 receptors [42]. Other pathways implicated in cardiomyogenic differentiation include the Nodal/Cripto/Alk4 signal pathway [57], JAK2/STAT3 [58], oxytocin/oxytocin receptor system [45], GTPase Rac1 [59], Wnt11 signaling [60], sP(3)-dependent Ca^{2+} signals [61], and protein kinase C signaling [46, 62]. Elucidation of the mechanisms underlying ES cell differentiation (both into cardiomyocytes and other cell types) will be an important requisite for optimizing potential therapies that might emerge from this field.

In vivo data from animal models

Data obtained from rodent models evaluating the fate of undifferentiated ES cells transplanted into the heart demonstrate mixed results. Early studies by Hodgson et al. and Xiao's groups evaluated mouse ES xenotransplantation into the hearts of immunocompetent rats following left anterior artery ligation induced infarction [63–65]. Collective data from these studies appeared quite encouraging. Investigators found evidence of long-term (12–32 weeks) survival of transplanted ES cells and, based on postmortem histological evaluation, differentiation of surviving cells into cardiac lineages. Cell therapy also mitigated postinfarct remodeling, improved angiogenesis within the infarct zone, and conferred an improvement in cardiac function compared to injection of saline or medium alone. Adverse sequelae such as graft rejection, sudden cardiac death, or arrhythmogenesis were not appreciated in these studies. Most notably, no tumorigenesis or teratoma formation was observed. By contrast, more recent studies by Cao et al. demonstrated that mouse ES cells transplanted into the noninjured hearts of athymic nude mice formed teratomas by as early as 3 weeks following transplant [34]. In these studies, ES cells were stably transfected with molecular imaging reporter genes, allowing for repeated visualization and quantification of cell proliferation and migration *in vivo* by multiple imaging modalities (Figure 11.1). The observed logarithmic increase in cell signal between 2 and 3 weeks following transplant was consistent with uncontrolled proliferation and teratoma formation and was confirmed by postmortem histological analysis. Similarly, in studies conducted earlier by Swijnenburg et al., undifferentiated mouse ES cells also formed teratomas in the recipient hearts [66]. Results from these studies further underscore the importance of isolating purified cardiomyocyte populations suitable for transplant.

To this end, several groups have now pushed ahead with experiments using small and large animal models for evaluation of ES-cell-derived cardiomyocytes transplanted into the heart. Laflamme et al. demonstrated that human ES-cell-derived cardiomyoblasts transplanted into athymic rat hearts successfully engraft, proliferate, and express several cardiac cell markers, without evidence of teratomas up to 4 weeks postoperatively [67]. Xue and colleagues transplanted cardiomyocytes derived from human ES cells into guinea pig hearts and found the cells to engraft as well [7]. Moreover, electrophysiological mapping of the hearts revealed that the transplanted cells functionally integrated

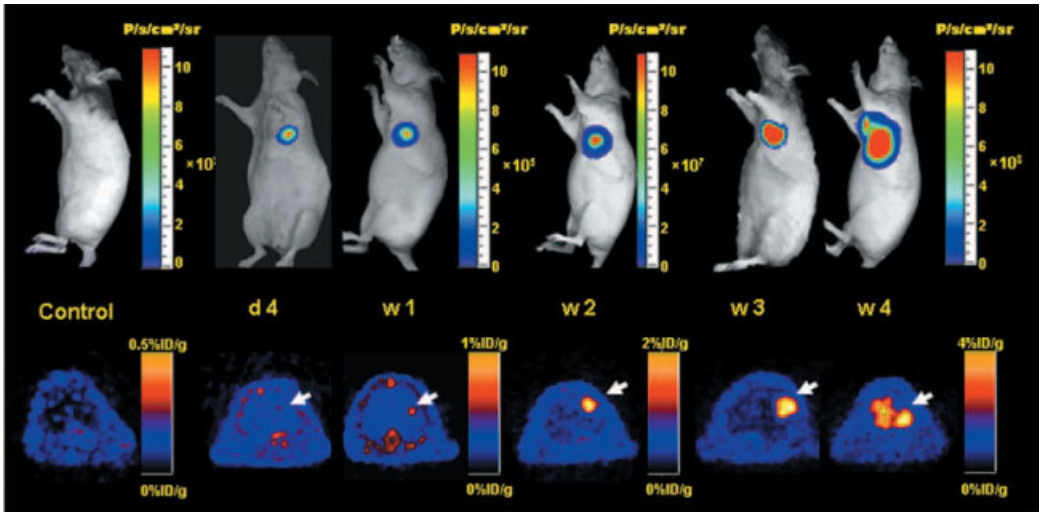


Figure 11.1 Multimodality molecular imaging of embryonic stem cells in living animals. Upper panel: optical bioluminescence imaging showing mouse ES cells expressing the Fluc gene at day 4, week 1, week 2, week 3, and week 4 after intramyocardial transplantation. Lower panel: detailed tomographic view of cardiac positron emission tomography (PET) imaging of [^{18}F]-FHBG activity, showing mouse ES cells expressing the HSV1-ttk (herpes simplex virus 1-truncated thymidine kinase) reporter gene at corresponding time points. (Adapted from [34], with permission.)

and electrocoupled with host myocardium. This work confirmed the finding presented earlier by Kehat et al., who demonstrated electromechanical and structural coupling of transplanted human ES-cell-derived cardiomyoblasts with the host myocardium in pigs [68] These collective data suggest that human ES-cell-derived cardiomyoblast transplantation might potentially be feasible and safe. However, as will be discussed, there still remain many hurdles and significant concerns before such therapy may even be considered in humans.

Keeping an eye on therapy: imaging of ES cells

As the field of ES cell transplant gradually translates into reality, it will become imperative that methodologies be adopted to monitor and assess the bioactivity and location of the transplanted cells. In presently established areas of transplant biology, both biochemical assays and imaging modalities exist to provide detailed information as to the structure and function of the transplanted organ. For example, a liver transplant can easily be assessed by a host of serum blood tests and various types of imaging (ultrasound, magnetic resonance imaging, computed tomography). However, assessing the status of stem cell transplant therapy is not so simple. There are presently no good direct measures of cell function that can be performed *in vivo* for humans. If one examines the experience with bone marrow stem cell therapy for the heart, for example, it becomes readily apparent that the nearly 20 clinical trials being conducted worldwide have failed to track the long-term fate of transplanted cells simply because there are no good tools to do so. Moreover, efficacy of the cell transplants is measured by assessment of heart function, but this data cannot be correlated to the biological state of the cells, since the cells themselves can neither be visualized nor assayed in the living subject. Although the appropriate methodologies and tools are not yet available in the clinic, they are available for use at the benchtop and for small-animal studies.

As ES cell biology is further elucidated in the laboratory setting, the need for concurrent development of methodologies to track and assess these cells *in vivo* should not be neglected. Studies by Wu's group at Stanford have demonstrated the importance of this concept as demonstrated by their finding that undifferentiated mouse ES cells transplanted into the donor heart result not only in local teratoma

formation, but also produce metastatic spread of the tumor [34]. Such a finding might have been more easily overlooked if assessment of the transplant was made by traditional, postmortem histological analysis alone. Instead, the ability to image the entire animal and track the whole body distribution of transplanted cells (and their progeny) allowed for continual, quantitative assessment of tumor load (Figure 11.2) as well as efficacy of antitumor therapy (discussed below).

A detailed discussion of the various molecular imaging techniques available to evaluate cell therapy is outside the scope of this chapter (for a comprehensive review, please refer to [69]). However, investigators have successfully used magnetic resonance imaging [70], positron emission tomography (PET) [34], and bioluminescent imaging [34] to track ES cell transplantation *in vivo*. It is likely that such technologies will help guide the development of clinically suitable human ES-cell-derived cardiomyocytes and will also be used to assess their clinical efficacy in the future.

Translation into the clinic: practical challenges

Although present data suggest that human ES-cell-derived cardiomyoblast transplantation may provide a reasonable means of myocardial restoration, there remain several unresolved issues and gaps to be bridged between the laboratory and bedside. Some of the major areas for concern regarding human ES cell therapy include the immunogenic profile of the cells (and their derivative lineages), potential for posttransplant cellular misbehavior (i.e., teratoma formation), optimizing cell delivery and retention, as well as potential for posttransplant arrhythmogenecity. These topics will be touched upon briefly.

Allogeneic rejection by recipients may severely hamper the therapeutic efficacy of transplanted ES cell (see Chap. 3). Whether ES cells are, in fact, immunoprivileged remains undetermined, with conflicting evidence by several investigators. Drukker et al. have shown that the immunostimulatory profile of human ES cells and their differentiated derivatives is quite mild, given their relatively low to absent levels of major histocompatibility complex and natural killer cell receptor expression [71]. Further

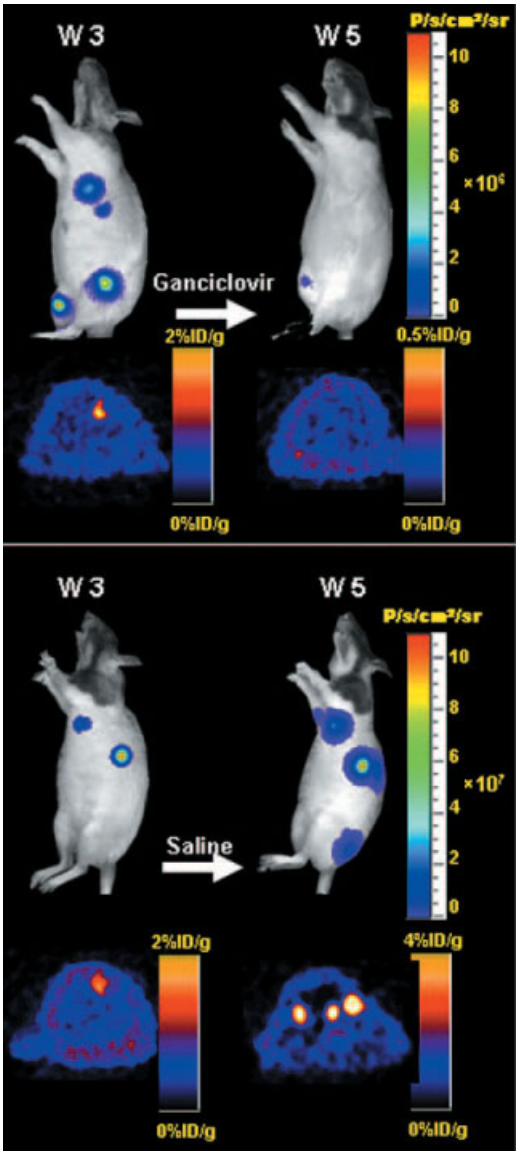


Figure 11.2 Ablation of teratoma formation with HSV1-ttk as both a reporter gene and a suicide gene. Treatment of control animals with saline resulted in multiple teratoma formation by week 5 (bottom panel). Treatment of study animals with ganciclovir for 2 weeks showed abrogation of both bioluminescence and PET imaging signals (top panel). (Adapted from [34], with permission.)

support for the immunoprivileged hypothesis is provided by data from Li et al., who showed that human ES cells injected into the quadriceps muscle of immunocompetent mice did not elicit an immune response as assayed by histological evaluation

[72]. Finally, Menard et al. demonstrated no survival advantage of mouse ES cells transplanted into the hearts of immunosuppressed sheep compared to those receiving no antirejection therapy at 1 month posttransplant [73]. In contrast to these findings, a series of studies from Robbins' group suggest that ES cells do mount a host immune response *in vivo*. Specifically, Kofidis et al. transplanted mouse ES cells into the hearts of syngeneic, allogeneic, and immunocompromised recipients [74]. Results from these studies showed that cellular transplant induced a humoral antibody and local cellular infiltrative immune response that was highest in the in the allogeneic recipients. Swijnenburg et al. also evaluated the immunogenicity of mouse ES cells *in vivo* by cardiac transplantation into syngeneic and allogeneic hosts [66]. These experiments revealed immune cell infiltration into the allogeneic graft sites, with no detectable transplanted cells at 8 weeks postoperatively. In contrast, the syngeneic hosts had persistent engraftment of transplanted cells at 8 weeks and exhibited no evidence of infiltration. Clearly, the immunogenicity of ES cells and their derivatives warrants further, in-depth investigation.

Another potential pitfall of ES cell therapy remains the potential for teratoma formation (see Chap. 3). Although it is clear that undifferentiated ES cells will give rise to teratomas *in vivo*, present data seem to suggest that pure populations of cardiomyocytes do not exhibit such cellular misbehavior. Regardless, contamination of pure populations with undifferentiated cells and/or dedifferentiation of cells to a more immature state are valid concerns. To date, no one has described a 100% pure population of ES-cell-derived cardiomyocytes. It will be essential to achieve pure populations of such cells if the threat of cellular misbehavior (i.e., teratoma formation) is to be minimized. Clearly, to realize the goal of clinical cellular transplantation, further refinement of the cardiomyocyte isolation and enrichment processes is absolutely needed. In addition, fail-safe measures to control cell behavior posttransplant may also be of utility. Such an approach has been already demonstrated in animal studies by Cao et al. [34]. To effectively curb teratomas arising from transplanted cells *in vivo*, this group utilized the concept of suicide gene therapy. To achieve this, a dual property of thymidine kinase (TK) reporter gene was exploited. ES cells were stably transfected with the viral TK gene prior to transplantation. This gene allows for visualization of the cells when a radioactively tagged PET reporter probe, 9-(4-fluoro-3-hydroxymethylbutyl) guanine ([^{18}F]-FHBG), is injected into the animal. Only ES cells expressing the TK reporter gene will phosphorylate and trap the [^{18}F]-FHBG PET reporter probe. However, TK also selectively phosphorylates antiviral drugs such as ganciclovir, allowing for targeted destruction of the transplanted cells if need be. This rather elegant system is illustrated in Figure 2, which demonstrates the utility of noninvasive imaging to track cell therapy and evaluate the efficacy of treatments designed to modulate cellular behavior *in vivo*.

The issues of cell delivery, retention by the myocardium, and posttransplant survival remain areas that require further investigation and optimization. For example, the ideal timing (acute or late after myocardial infarction) and delivery routes (intracoronary, direct myocardial injection, or systemic administration) needed for optimal cell engraftment and retention still need to be determined. Additionally, the degree of injury of the host myocardium will likely affect the ability of cells to acutely engraft. This has been demonstrated by Dow and colleagues who have shown in rats that ischemia reperfusion injury mitigates transplanted cell retention compared to hearts that underwent left anterior artery ligation [75]. Such findings point to the importance of the host environment in supporting cellular grafts. One of the key challenges in this regard will remain optimizing vascular support for freshly engrafted cells. Investigators have postulated that a possible reason for the early demise of cellular grafts may largely be due to inadequate angiogenic support. A potential solution may lie in the concomitant introduction of vascular progenitor cells that might proliferate in concert with the cell graft to achieve an adequate vascular support structure (see Chaps 12 & 13). The source of such cells may be from circulating progenitor cells or endothelial progenitors derived from ES cell themselves. Alternatively, "pretreatment" or adjuvant angiogenic gene therapy to optimize peritransplant angiogenesis may also be avenues to pursue. The need to further advancement in this area is clear and will likely be integral to the advancement of cardiac cellular transplantation.

Ultimately, prolonging the survival of transplanted cell grafts will likely involve optimization

of various biological systems, including modulation of the immune response, regulation of cell homeostasis mechanisms, providing bioartifical cell scaffolds, establishing adequate vascular support, and balancing cell apoptotic pathways. The latter strategy has already been explored *in vivo* by Kutschka et al., who showed that cardiomyocytes overexpressing the antiapoptotic gene BCL-2 had prolonged survival compared to control cardiomyocytes transplanted into infarcted rat hearts [76]. Such approaches may also benefit transplanted ES cell survival in the future.

Although arrhythmogenicity has not yet manifested as a potential complication of ES-cell-derived cardiomyocyte transplantation in animal models, such cells have demonstrated arrhythmic potential *in vitro* [77]. To prevent ectopic foci of cardiac electrical activity from forming, measures will be needed to ensure appropriate host–donor electromechanical coupling. These technologies remain to be described. Finally, genetic manipulation of the cells prior to transplant such that selective "electric gating" or quiescence might be accomplished posttransplant by administration of a drug or small molecule may also be another fail-safe feature.

Ethical considerations

No discussion of ES cell research would be complete without a mention of the politico-ethical debate the field has spawned. Without a doubt, ES-cell-related research holds great potential for millions suffering from numerous of medical conditions, including heart disease. However, as the only source for new human ES cell lines remains human embryos, the issue remains charged, with many opposed to the idea of using cells derived from such a source. In the United States, the debate has bled over from the confines of ethico-religious circles and has become a galvanized political issue [78], with the present administration staunchly fixed against either expanding funding for or supporting the development of novel ES cells lines. This has placed the responsibility of charting new frontiers in the hands of the private sector in the United States as well as academic centers and industry based in other nations that are more aggressively pursuing the promising area of ES cell research.

Conclusion

The last several years have produced revolutionary advancements in the area of ES cell research. These unique cells may potentially change the face of regenerative medicine as well as provide profound insights into the mechanisms of development and disease progression. With regards to myocardial restorative therapy, initial results appear promising, as ES-cell-derived, differentiated cardiomyocytes seem to improve myocardial function following injury. Nonetheless, we are still many years away from safely translating these initial observations into therapy for humans. To date, ES cells have not been delivered to humans. It is likely that the initial patient populations that will most benefit from such treatment will be those suffering from debilitating, degenerative disease processes such as chronic heart failure and neurological disorders. If such goals are to be achieved, however, they will be predicated upon a detailed understanding and appreciation for the biology and mechanistic details underlying ES cell growth, development, function, differentiation, and survival.

References

1 Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000; 227(2):271–278.

2 Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282(5391):1145–1147.

3 Thomson JA, Marshall VS. Primate embryonic stem cells. Curr Top Dev Biol 1998; 38:133–165.

4 Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001; 108(3):407–414.

5 He JQ, Ma Y, Lee Y, et al. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res 2003; 93(1): 32–39.

6 Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002; 91(6):501–508.

7 Xue T, Cho HC, Akar FG, et al. Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the

development of cell-based pacemakers. Circulation 2005; 111(1):11–20.
8 Mummery C, Ward-van Oostwaard D, Doevendans P, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 2003; 107(21):2733–2740.
9 Solter D, Knowles BB. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci USA 1975; 72(12):5099–5102.
10 Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 1981; 78(12):7634–7638.
11 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292(5819):154–156.
12 Reubinoff BE, Pera MF, Fong CY, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; 18(4):399–404.
13 Carpenter MK, Rosler E, Rao MS. Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells 2003; 5(1):79–88.
14 Guhr A, Kurtz A, Friedgen K, Loser P. Current state of human embryonic stem cell research: an overview of cell lines and their usage in experimental work. Stem Cells 2006; 24(10): 2187–91.
15 Xu C, Inokuma MS, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001; 19(10):971–974.
16 Pease S, Braghetta P, Gearing D, et al. Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Dev Biol 1990; 141(2):344–352.
17 Beattie GM, Lopez AD, Bucay N, et al. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem Cells 2005; 23(4):489–495.
18 Xie X, Chan RJ, Yoder MC. Thrombopoietin acts synergistically with LIF to maintain an undifferentiated state of embryonic stem cells homozygous for a Shp-2 deletion mutation. FEBS Lett 2002; 529(2–3):361–364.
19 Rose TM, Weiford DM, Gunderson NL, Bruce AG. Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells in vitro. Cytokine 1994; 6(1):48–54.
20 Wang L, Li L, Menendez P, et al . Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development. Blood 2005; 105(12):4598–4603.
21 Xu RH, Peck RM, Li DS, et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2005; 2(3):185–190.
22 Xu C, Rosler E, Jiang J, et al. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 2005; 23(3):315–323.
23 Ginis I, Luo Y, Miura T, et al. Differences between human and mouse embryonic stem cells. Dev Biol 2004; 269(2):360–380.
24 Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. Stem Cells 2001; 19(3):193–204.
25 Yoo SJ, Yoon BS, Kim JM, et al. Efficient culture system for human embryonic stem cells using autologous human embryonic stem cell-derived feeder cells. Exp Mol Med 2005; 37(5):399–407.
26 Richards M, Fong CY, Chan WK, et al. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002; 20(9):933–936.
27 Xu C, Jiang J, Sottile V, et al. Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. Stem Cells 2004; 22(6):972–980.
28 Genbacev O, Krtolica A, Zdravkovic T, et al. Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. Fertil Steril 2005; 83(5):1517–1529.
29 Amit M, Margulets V, Segev H, et al. Human feeder layers for human embryonic stem cells. Biol Reprod 2003; 68(6):2150–2156.
30 Cheng L, Hammond H, Ye Z, et al. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells 2003; 21(2):131–142.
31 Rosler ES, Fisk GJ, Ares X, et al. Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn 2004; 229(2):259–274.
32 Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 2004; 70(3):837–845.
33 Klimanskaya I, Chung Y, Meisner L, et al. Human embryonic stem cells derived without feeder cells. Lancet 2005; 365(9471):1636–1641.
34 Cao F, Lin S, Xie X, et al. In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. Circulation 2006; 113(7):1005–1014.
35 Martin GR, Evans MJ. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc Natl Acad Sci USA 1975; 72(4):1441–1445.
36 Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 1982; 94(2):253–262.
37 Passier R, Oostwaard DW, Snapper J, et al. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 2005; 23(6):772–780.
38 Baharvand H, Azarnia M, Parivar K, Ashtiani SK. The effect of extracellular matrix on embryonic stem

cell-derived cardiomyocytes. J Mol Cell Cardiol 2005; 38(3):495–503.

39 Pal R, Khanna A. Role of hepatocyte-like cells in the differentiation of cardiomyocytes from mouse embryonic stem cells. Stem Cells Dev 2005; 14(2):153–161.

40 Kawai T, Takahashi T, Esaki M, et al. Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2. Circ J 2004; 68(7):691–702.

41 Sachinidis A, Gissel C, Nierhoff D, et al. Identification of plateled-derived growth factor-BB as cardiogenesis-inducing factor in mouse embryonic stem cells under serum-free conditions. Cell Physiol Biochem 2003; 13(6):423–429.

42 Behfar A, Zingman LV, Hodgson DM, et al. Stem cell differentiation requires a paracrine pathway in the heart. FASEB J 2002; 16(12):1558–1566.

43 Singla DK, Sun B. Transforming growth factor-beta2 enhances differentiation of cardiac myocytes from embryonic stem cells. Biochem Biophys Res Commun 2005; 332(1):135–141.

44 Wu H, Lee SH, Gao J, et al. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 1999; 126(16):3597–3605.

45 Paquin J, Danalache BA, Jankowski M, et al. Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. Proc Natl Acad Sci USA 2002; 99(14):9550–9555.

46 Ventura C, Zinellu E, Maninchedda E, Maioli M. Dynorphin B is an agonist of nuclear opioid receptors coupling nuclear protein kinase C activation to the transcription of cardiogenic genes in GTR1 embryonic stem cells. Circ Res 2003; 92(6):623–629.

47 Takahashi T, Lord B, Schulze PC, et al. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 2003; 107(14):1912–1916.

48 Kanno S, Kim PK, Sallam K, et al. Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. Proc Natl Acad Sci USA 2004; 101(33):12277–12281.

49 Wobus AM, Kaomei G, Shan J, et al. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. J Mol Cell Cardiol 1997; 29(6):1525–1539.

50 Honda M, Hamazaki TS, Komazaki S, et al. RXR agonist enhances the differentiation of cardiomyocytes derived from embryonic stem cells in serum-free conditions. Biochem Biophys Res Commun 2005; 333(4):1334–1340.

51 Zandstra PW, Bauwens C, Yin T, et al. Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng 2003; 9(4):767–778.

52 Illi B, Scopece A, Nanni S, et al. Epigenetic histone modification and cardiovascular lineage programming in mouse embryonic stem cells exposed to laminar shear stress. Circ Res 2005; 96(5):501–508.

53 Sauer H, Rahimi G, Hescheler J, Wartenberg M. Effects of electrical fields on cardiomyocyte differentiation of embryonic stem cells. J Cell Biochem 1999; 75(4):710–723.

54 Zweigerdt R, Burg M, Willbold E, et al. Generation of confluent cardiomyocyte monolayers derived from embryonic stem cells in suspension: a cell source for new therapies and screening strategies. Cytotherapy 2003; 5(5):399–413.

55 Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 1996; 98(1):216–224.

56 Muller M, Fleischmann BK, Selbert S, et al. Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB J 2000; 14(15):2540–2548.

57 Parisi S, D'Andrea D, Lago CT, et al. Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells. J Cell Biol 2003; 163(2):303–314.

58 Foshay K, Rodriguez G, Hoel B, et al. JAK2/STAT3 directs cardiomyogenesis within murine embryonic stem cells in vitro. Stem Cells 2005; 23(4):530–543.

59 Puceat M, Travo P, Quinn MT, Fort P. A dual role of the GTPase Rac in cardiac differentiation of stem cells. Mol Biol Cell 2003;14(7):2781–2792.

60 Terami H, Hidaka K, Katsumata T, et al. Wnt11 facilitates embryonic stem cell differentiation to Nkx2.5-positive cardiomyocytes. Biochem Biophys Res Commun 2004; 325(3):968–975.

61 Puceat M, Jaconi M. Ca^{2+} signalling in cardiogenesis. Cell Calcium 2005; 38(3–4):383–389.

62 Ventura C, Zinellu E, Maninchedda E, et al. Protein kinase C signaling transduces endorphin-primed cardiogenesis in GTR1 embryonic stem cells. Circ Res 2003; 92(6):617–622.

63 Min JY, Yang Y, Converso KL, et al. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. J Appl Physiol 2002; 92(1):288–296.

64 Min JY, Yang Y, Sullivan MF, et al. Long-term improvement of cardiac function in rats after infarction by transplantation of embryonic stem cells. J Thorac Cardiovasc Surg 2003; 125(2):361–369.

65 Hodgson DM, Behfar A, Zingman LV, et al. Stable benefit of embryonic stem cell therapy in myocardial infarction. Am J Physiol Heart Circ Physiol 2004; 287(2):H471–H479.

66 Swijnenburg RJ, Tanaka M, Vogel H, et al. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 2005; 112(9 suppl):I166–172.

67 Laflamme MA, Gold J, Xu C, et al. Formation of human myocardium in the rat heart from human embryonic stem cells. Am J Pathol 2005; 167(3):663–671.

68 Kehat I, Khimovich L, Caspi O, et al. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nat Biotechnol 2004; 22(10):1282–1289.

69 Sheikh AY, Wu JC. Molecular imaging of cardiac stem cell transplantation. Curr Cardiol Rep 2006; 8(2):147–154.

70 Arai T, Kofidis T, Bulte JW, et al. Dual in vivo magnetic resonance evaluation of magnetically labeled mouse embryonic stem cells and cardiac function at 1.5 t. Magn Reson Med 2006; 55(1):203–209.

71 Drukker M, Katz G, Urbach A, et al. Characterization of the expression of MHC proteins in human embryonic stem cells. Proc Natl Acad Sci USA 2002; 99(15):9864–9869.

72 Li L, Baroja ML, Majumdar A, et al. Human embryonic stem cells possess immune-privileged properties. Stem Cells 2004; 22(4):448–456.

73 Menard C, Hagege AA, Agbulut O, et al. Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. Lancet 2005; 366(9490):1005–1012.

74 Kofidis T, deBruin JL, Tanaka M, et al. They are not stealthy in the heart: embryonic stem cells trigger cell infiltration, humoral and T-lymphocyte-based host immune response. Eur J Cardiothorac Surg 2005; 28(3):461–466.

75 Dow J, Simkhovich BZ, Kedes L, Kloner RA. Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. Cardiovasc Res 2005; 67(2):301–307.

76 Kutschka I, Kofidis T, Chen IY, et al. Adenoviral human BCL-2 transgene expression attenuates early donor cell death after cardiomyoblast transplantation into ischemic rat hearts. Circulation 2006; 114(1 suppl):I174–180.

77 Zhang YM, Hartzell C, Narlow M, Dudley SC, Jr. Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. Circulation 2002; 106(10):1294–1299.

78 Jain KK. Ethical and regulatory aspects of embryonic stem cell research. Expert Opin Biol Ther 2005; 5(2):153–162.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

12 CHAPTER 12

Bone-marrow-derived cells in myocardial repair and regeneration

Raj Kishore, PhD, *& Douglas W. Losordo,* MD

Introduction

Despite the improvement shown in the prognosis of patients with acute myocardial infarction (AMI) with the use of available therapies including thrombolysis and urgent coronary revascularizations, there remains still a significant mortality risk while a significant proportion of MI survivors are at risk for developing heart failure. Post-MI left ventricular (LV) remodeling—a process characterized by a mechanical expansion of the infarcted wall followed by progressive LV dilation and dysfunction [1]—plays an important role in the progressive nature of postinfarct heart failure (see Chap 21). Since the main underlying trigger of this process is the loss of cardiomyocytes and microvasculature in the infarcted wall, treatment strategies aimed at preserving or regenerating myocardial tissue are currently being examined as potential therapeutic modalities.

In 1960, McCulloch and Till introduced the concept of the adult stem cell by identifying the bone marrow (BM) as a repository of cells having the capability of reconstituting the entire hematopoietic system following lethal irradiation [2]. Over 30 years later Asahara et al extended this concept, revealing that BM-derived cells were also capable of vasculogenesis (see Chap 5), a process previously considered to be restricted to embryonic life [3]. Evidence has also continued to accumulate, indicating the remarkable ability of adult stem cells to produce differentiated cells from embryologically unrelated tissues. There are also convincing data regarding the existence of cardiac stem cells, supporting the notion that the heart has its own intrinsic regenerative capacity [4]. In addition, both cardiac stem cells and extracardiac BM/BM-derived progenitors have been shown to regenerate myocytes and vasculature [5–9]. In particular, when BM-derived cells, both of murine and of human origin, are transplanted or mobilized in animal models of MI, they have been shown to home to infarct and periinfarct myocardium, inducing both myogenesis and angiogenesis, while improving cardiac function and survival [10]. Thus, ample experimental evidence supports the notion that transplanted BM-derived cells participate directly and indirectly in the regeneration of cardiac myocytes and the microvasculature post-MI.

Three available lines of evidence converge in favor of this interpretation: (1) the recent observations that cardiac endogenous stem cells are present in the normal myocardium (see Chap 9) and are involved in the maintenance of cardiac cellular homeostasis, with the ability to expand and regenerate myocytes and microvasculature in the infarcted myocardium [4], (2) the evidence that in humans cardiomyocyte repopulation by BM-derived progenitors of hematopoietic origin can take place [11, 12], and (3) the demonstration that it is possible to increase the efficiency of the intrinsic cardiac regenerative capacity of animals with AMI by the local delivery or mobilization of BM-derived cells, resulting in a reduction of infarct size and an improvement in LV performance and survival [5, 13–15]. It is therefore, reasonable to propose that BM-derived cells are part of an endogenous repair/regeneration process. These observations also provide evidence that BM-derived cells act through a combination of paracrine effects that stimulate the expansion, homing, and differentiation of endogenous cardiac

stem cells on one hand and the transdifferentiation of the BM-derived cells toward cardiac and vascular cells on the other [9, 16, 17]. In this chapter, we will present and discuss the preclinical and clinical evidence for myocardial/vascular regeneration limited to that derived from human BM-derived cells.

Human BM cell transplantation and cardiac repair: functional and anatomical evidence from preclinical studies

BM remains the best described source of adult stem cells, both in animals and in humans. Three stem cell subsets from the BM have been described, which include (i) hematopoietic stem cells (HSCs), characterized by the marker protein CD34 or the more immature marker protein CD133, and their peripheral blood/BM-derived progeny endothelial progenitor cells (EPCs) which also express endothelial markers (such as Flk1) along with CD34/CD133 [3, 10] (see also Chap 5) (ii) a specific subset of enriched HSCs termed as "side population" (CD34−/low, c-Kit+, Sca-1+lin−) cells [18] (see also Chapter 7); and (iii) mesenchymal stem cells (MSCs) which are characterized by the expression of various cell surface markers depending on origin and *in vitro* expansion procedures [19] (see also Chap 4). A subset of MSCs, multipotent adult progenitor cells, has also been described which differentiate, at the single-cell level, not only into mesenchymal cells but also into cells with characteristics of the visceral mesoderm, neuroectoderm, and endoderm *in vitro* [20]. Our laboratory has also described another multipotent BM-derived cell notable for the lack of surface markers that had characterized previously described cells [8].

Most preclinical data using human BM/peripheral blood-derived progenitor cell transplantation in the setting of myocardial ischemia in small animals are limited to HSCs/EPCs and MSCs, and form the basis of the following discussion. Table 12.1 summarizes the existing preclinical evidence regarding the postinfarct myocardial repair that follows transplantation of human BM-/peripheral blood-derived cells [5, 8, 13, 21–28].

An integral component of the repair process is neovascularization within the myocardial infarct and periinfarct regions. The results of preclinical studies have consistently shown the relationship between the increases in capillary density and the preservation of function. Interventions post-MI that increase or preserve the microcirculation also appear to improve myocardial function. The isolation, characterization, and functional contribution of BM-derived circulating EPCs in the process of ischemic neovascularization was first described in our laboratories [3]. These findings led to a number of subsequent follow-up studies using human EPC transplantation in athymic rodents and have demonstrated repeatedly that therapeutic neovascularization and functional blood flow recovery occurred in surgically induced hind limb ischemia models [29, 30]. Human BM-derived cells have also been used for the repair of experimental MI in small animals. Granulocyte-colony-stimulating factor mobilized human BM-derived CD34+ cells, when implanted in immunodeficient rats after experimental MI, were shown to directly induce new blood vessel formation in the infarct bed and to induce proliferation of the preexisting vasculature. The neovascularization resulted in a decreased apoptosis rate of hypertrophied myocytes in the periinfarct region, the long-term salvage and survival of viable myocardium, a reduction in collagen deposition, and a sustained improvement in cardiac function [13]. Similarly, studies from our laboratories revealed that transplantation of *ex vivo* expanded human EPCs in a rat model of AMI enhanced myocardial capillary density, led to the incorporation of transplanted human EPCs into foci of myocardial neovascularization, and had a favorable impact on the preservation of LV function [5].

These preclinical data are consistent with clinical data, indicating that the microvascular perfusion post-MI, whether assessed by contrast echo [31] or angiography [32], correlates closely with the outcome. Most notably, and consistent with preclinical data, the pilot clinical trial data in patients have shown that microvascular function, measured as coronary flow reserve, increases after administration of BM-derived progenitor cells to patients and parallels clinical improvement [33].

Another integral component of the remodeling process in the infarcted heart is characterized by progressive expansion of the initial infarct area and dilation of the LV lumen with cardiomyocyte replacement by fibrous tissue deposition in the

Table 12.1 Experimental studies using human bone-marrow-derived stem/progenitor cells in animal models of myocardial infarction.

Model/study	*Cells*	*Route of cell application*	*Functional/histological outcome*
SCID mouse MI [21]	BM-derived MSC	Intramuscular injection	Decreased infarct size, increased capillary density, improved ventricular function, transplanted cells positive for cardiac markers
Sheep *in utero* [22]	BM-MSC	5–20 $\times$ 10^6 cells/fetus intraperitoneal	Long-term engraftment and site-specific differentiation into chondrocytes, adipocytes, myocytes, and cardiomyocytes of transplanted human cells.
In vitro [23]	BM mononuclear cells	Seeded on mouse heart slices and *ex vivo* cultured	Seeded human BM cells acquired cardiomyocyte phenotype and expressed cardiac proteins.
MI in athymic nude rats [13]	G-CSF-induced freshly isolated peripheral CD34 + cells	Intravenous injection of 2 $\times$ 10^6 cells	Stimulation of neovascularization. Preservation of LV function
MI in athymic nude rats [5]	Peripheral Blood EPCs	Intravenous injection of 10^6 culture-expanded human EPCs	Improved cardiac hemodynamics. Increased capillary density. Decreased ventricular scarring
Nude rat AMI [8]	Clonally expanded BM stem cells	8 $\times$ 10^5 hBMSCs, intramyocardial injections	Improved ventricular functions, transdifferentiation into endothelial, smooth muscle and cardiomyocytes *in vivo*
SCID mice MI [24]	Peripheral blood CD34+ cells	Intramyocardial injections	Cardiomyocyte differentiation of CD34+ cells by cell fusion and to EC by transdifferentiation
NOD/SCID mice MI [25]	Cord blood mononuclear cells	Tail-vein injections	Homing to ischemic heart, smaller infarct area, higher capillary density, no CM differentiation, chimeric capillaries
SCID mice MI [26]	Peripheral blood CD34+ cells	Intramyocardial injections	Cardiomyocyte and endothelial transdifferentiation, cell fusion
CB17 SCID/*beige* mice heart [27]	BM-MSCs	Intracardiac injection cells	Differentiation of engrafted MSCs into cardiomyocytes
SCID mice MI [28]	Human peripheral blood CD34+ cells	Intravenous	Engraftment and injury induced CM, EC, SMC transdifferentiation

SCID = *severe combined immunodeficiency*; NOD = *nonobese diabetic*; AMI = acute myocardial infarction; BM-MSC = bone marrow mesenchymal stem cell; G-CS = granulocyte colony-stimulating factor; LV = left ventricular ; EPCs = endothelial progenitor cells; hBMSCs = human bone marrow stem cells; CM = *cardiomyocytes*; EC = endothelial cell; SMC = smooth muscle cell.

ventricular wall. As discussed above, neovascularization induced by BM-derived cells represents one aspect of cardiac repair that can result in the preservation of cardiac function. A complementary approach to reverse myocardial remodeling is to use BM-derived stem cells for the regeneration of cardiac myocytes. The capability of a subpopulation of BM cells to differentiate into cardiomyocytes is a crucial but controversial biological issue. Several experimental reports have documented that new

myocytes and vascular cells are formed from labeled cells injected into damaged myocardium [4, 8, 14, 15, 34]. Clinical studies, particularly of heart and BM sex-mismatched organ transplants in humans, have provided clear evidence to support this paradigm [6]. In contrast to these findings, other investigators have been unable to document myocyte differentiation in the mouse model and, therefore, question the potential of BM cells to regenerate myocardial tissue [35, 36].

Most of the evidence regarding cardiomyocyte differentiation of transplanted BM cells or, for that matter the lack thereof, come from either genetically or chemically tagged syngeneic BM cell transplantation studies in small animal models of experimental AMI. However, there does exist some evidence that when human BM-derived cells are transplanted in AMI models of immunodeficient rodents, they will acquire phenotypic characteristics consistent with cardiomyocyte identity. Human BM-derived MSCs, for example, when transplanted *in utero* in sheep, show engraftment and site-specific differentiation, including that of cardiomyocytes [22]. In another study, labeled human BM cells, when seeded on top of cryoinjured mice heart slices and further cultured *in vitro*, demonstrated tropism for and ability to graft into the damaged mouse cardiac tissue. After 1 week the cells acquired a cardiomyocyte phenotype and expressed cardiac proteins, including connexin 43 [23]. It should also be noted that multipotent cells, for example, MSCs, have been identified in adult human BM and can be induced, under appropriate culture conditions, to differentiate into lineages of diverse tissues such as bone, cartilage, fat, tendon, and both skeletal and cardiac muscle [37].

One question often raised and debated is the plasticity of human BM-derived cells toward multilineage transdifferentiation, especially *in vivo*. Our recent study addresses many of these concerns [8]. We were able to identify a subpopulation of stem cells within the adult human BM, isolated at the single-cell level, that can self-renew without loss of multipotency for more than 140 population doublings and exhibits a capacity for differentiation into cells of all three germ layers. Based on the analysis of surface marker expression, it maybe said that these clonally expanded human BM-derived multipotent stem cells do not appear to belong to any previously described BM-derived stem cell population. Intramyocardial transplantation of human BM-derived multipotent stem cells after MI resulted in a robust engraftment of transplanted cells, which exhibited colocalization with markers of cardiomyocyte, endothelial cells (ECs), and smooth muscle cell identity (Figure 12.1), consistent with the differentiation of human BM-derived stem cells into multiple lineages found in *in vitro* studies. Our studies have provided extensive hemodynamic evidence from both autologous and heterologous BM-derived cell transplantation of an improvement in ventricular function in the setting of AMI. In addition, they provide evidence that human BM-derived cells are capable of transdifferentiation into cardiomyocytes, ECs, and vascular muscle cells, *in vivo*.

Human BM cell transplantation and cardiac repair: mechanistic evidence

Most of the experimental data describing the mechanistic aspects of BM-derived stem-cell-mediated cardiac repair have come from small animal AMI studies using either injections of chemically tagged, *ex vivo* expanded BM/peripheral blood-derived autologous cells (for example, CD34+ cells and EPCs) or from studies using syngeneic genetically tagged BM transplantations. The available evidence from small animal MI studies using human BM-derived cells generally shows a similar trend. Stem/progenitor cells may contribute to cardiac repair/regeneration in several ways. This may include at least two critical processes; physical transdifferentiation of transplanted cells into desired cell type, resulting in the replacement of lost specific cells, for example, cardiomyocytes and ECs (often referred as *hardware*) and by paracrine effects via secretion of angiogenic, cell survival cytokines/growth factors (often referred as *software*). Some of the involved mechanisms of BM-derived stem-cell-mediated myocardial repair are discussed below.

Enhanced neovascularization

It has been demonstrated that BM-derived EPCs circulate in the peripheral blood [3, 38], home to and incorporate into foci of neovascularization in

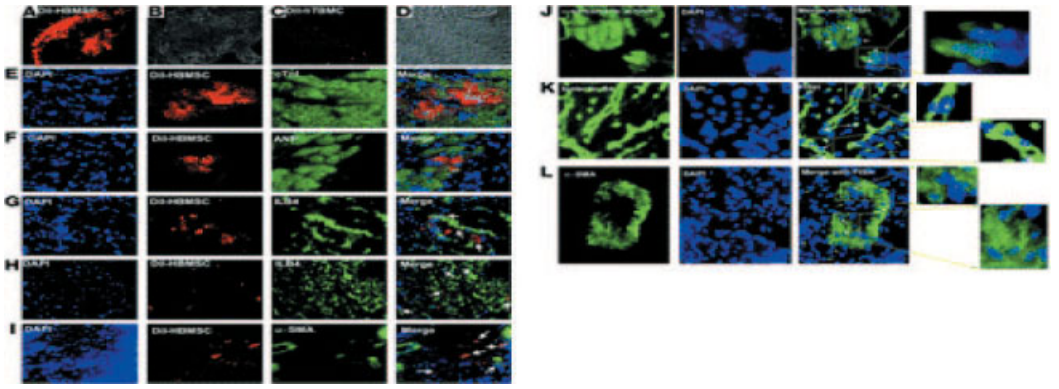


Figure 12.1 Engraftment and multilineage differentiation of transplanted human bone marrow stems cells (hBMSCs) in infarcted myocardium. (A–D) Engraftment of DiI-labeled hBMSCs and transplanted bone marrow cells (TBMCs) into infarcted myocardium. Numerous hBMSCs (red fluorescence) are engrafted into the infarct and periinfarct region of myocardium at 4 weeks after transplantation (A). In contrast, considerably fewer TBMCs (red fluorescence) are observed, mostly within the infarct area (C). (B) and (D) are the Hoffman images of (A) and (C), respectively, showing the localization of engrafted cells. (E and F) Immunophenotypic characterization of hBMSCs that have differentiated into cardiomyocytes (CMCs). Myocardial samples 4 weeks after transplantation were stained for cTnI (E) and ANP (F) (each detected with FITC-labeled secondary Ab). Transplanted DiI-hBMSCs expressed both markers and were indistinguishable from host CMCs. (G and H) Myocardial sections stained with ILB4—an endothelial cell (EC) marker—demonstrate that DiI-hBMSCs are colocalized with vascular ECs in both the infarct (G) and the periinfarct (H) area (arrows). (I) Myocardial sections stained with α-sarcomeric actinin (α-SMA) illustrate DiI-hBMSC colocalized with vascular smooth muscle cells (SMCs) (arrows). (J–L) Fluorescence *in situ* hybridization (FISH) on hBMSC transplanted hearts. FISH with α-SMA staining (J) demonstrates that transplanted hBMSCs shown in FISH+ red fluorescence express a CMC phenotype (green fluorescence). FISH with ILB4 staining (K) demonstrates that transplanted hBMSCs shown in FISH+ red fluorescence exhibit a vascular EC phenotype (green fluorescence). FISH with α-SMA staining (L) reveals that transplanted hBMSCs shown in FISH+ red fluorescence express an SMC phenotype (green fluorescence). White arrows indicate FISH+ cells stained with CMC, EC, or SMC markers; yellow arrows indicate FISH+ cells not stained with CMC, EC, or SMC markers. Scale bars in (A–I): 100 μm; scale bars in (J–L): 50 μm.

adult animals [39], and increase in number in response to tissue ischemia [40]. A large number of studies in rodents using autologous cells, syngeneic or allogeneic EPCs in the setting of hind limb [29, 30], or myocardial ischemia [5, 13, 41] have documented an enhancement of neovascularization (see Chapter 11). With regard to human BM/peripheral blood-derived EPC transplantation in murine AMI models, systemic administration of *ex vivo* expanded EPCs led to their incorporation into foci of myocardial neovascularization and had a favorable impact on the preservation of LV function [5]. Direct intramyocardial injection of human CD34+ cells has also been shown to induce new blood vessel formation in the infarct vascular bed and proliferation of the preexisting vasculature after an experimental MI in rats [13, 24, 28], while mobilization and recruitment of CD34+ cells from the marrow results in enhanced proliferation and incorporation into the myocardial microvasculature, even at remote time points post-MI [42]. Similarly, transplantation of either human BM-derived MSCs (Table 12.1) or human BM-derived multipotent stem cells [8] in AMI murine models was shown to improve myocardial neovascularization, as measured by increased capillary density.

Transdifferentiation and/or cell fusion

Transplantation of various BM-derived stem cells, both of rodent and human origins, after an MI leads to an engraftment of these stem cells in the periinfarct area. When human genetic markers or dye-labeled human BM-derived stem/progenitor cells were transplanted in mice/rat AMI models and tracked *in vivo*, the engrafted cells expressed endothelial and/or cardiomyocyte marker proteins [5, 8, 13, 16, 24, 27, 28]. These studies provide evidence of the hardware component of BM-derived

cell therapy and indicate that transplanted cells can differentiate into multiple phenotypes within the myocardium, thereby contributing to myocardial regeneration.

Other studies from murine BM cell models have suggested that the phenotypic changes of BM stem cells may occur as a consequence of cell fusion rather than transdifferentiation [43, 44]. However, the interpretation of these study results has been questioned based upon the recent finding of nanotubes that can transfer organelles and proteins between cells, providing the appearance of fusion [45]. It is noteworthy that under normal conditions many cardiomyocytes have two or more nuclei [46], suggesting that cell fusion may occur naturally. Therefore, genetic material derived from BM stem cells may contribute through cell transfer or fusion to the survival and function of cells within the myocardium.

Paracrine effects

In contrast to the restoration of lost myocardial cells by transdifferentiation of transplanted BM stem cells, the paracrine or so-called software effect of these cells is less equivocal. BM-derived stem and progenitor cells have been consistently shown to home to the sites of ischemia. This allows the local release of factors acting in a paracrine manner on the surrounding ischemic tissue. BM-derived mononuclear cells release angiogenic growth factors such as vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor, and angiopoietins, thereby enhancing the local angiogenic response and cardiomyocyte survival [8, 47].

Despite the noted variability in the mechanistic actions of transplanted BM-derived cells for myocardial repair or regeneration, we would like to mention that the term *myocardial regeneration* should be used for the convenience of understanding the global therapeutic effects of BM cells in various myocardial diseases. The term *myocardial repair or regeneration* encompasses a broad range of phenomena. Fundamentally, it refers to the generation of new cells or tissues that are deficient in the myocardium. However, in much of the literature related to stem cells, it may also be used to describe salvage of endangered myocardial cells. This variability arises from the disease models targeted by the stem cell therapy in individual investigations. Most of the investigations on the therapeutic use of stem cells, including those derived from BM, have been performed with animal models of AMI, in which the cells were transplanted within a few hours of MI—a time point at which the process of myocardial cell loss and replacement fibrosis is not completed. Therefore, it must be acknowledged that the myocardial regeneration/repair observed in most of this literature describes multiple components, such as the generation of new cells as well as the rescue of the degenerating cells in the myocardium.

Human BM cell transplantation: experience from clinical trials

On the basis of the above premises and data obtained from preclinical studies, a number of clinical trials using autologous BM/peripheral as elsewhere peripheral blood-derived stem/progenitor cells have been completed or are currently underway for postinfarct myocardial repair. In the clinical setting, the effect of BM-derived cells or peripheral blood-derived EPCs has also been assessed (Table 12.2) [33, 48–56]. BM cells (mean number 2.8×10^7) were infused 5–9 days after an AMI. In comparison with ten nonrandomized control patients who did not undergo BM aspiration or additional catheterization, BM cell infusion enhanced regional perfusion in the infarct region as assessed by thallium scintigraphy. Moreover, hemodynamic variables including stroke volume, end systolic volume, and regional contractility indices were improved after cell therapy [48]. In TOPCARE-AMI, BM-derived or peripheral blood EPCs were infused after a mean of 5 days post-MI. The intervention was shown to improve global ejection fraction as assessed by LV angiography, compared to no change in a nonrandomized control patient population [33]. The functional improvement and reduction of end systolic volumes were documented by magnetic resonance imaging in a patient subgroup [55]. Cell therapy also increased coronary flow reserve, which indicates an enhancement of neovascularization. The effects of EPCs from BM and peripheral blood were comparable, suggesting that both cell types have the capacity to augment cardiac regeneration.

In addition, these findings have been replicated in a randomized controlled clinical trial [54] of 60 patients with AMI, who were randomized to

Table 12.2 Clinical trials using bone-marrow-derived stem/progenitor cells in acute and chronic MI.

Disease/study	*Cell source*	*Route of cell application*	*Outcome*
AMI (20 pts) [48]	BM-MNCs (10 pts), no cell infusion (10 pts)	Intracoronary infusion during PTCA	Reduced infarcted area; improved cardiac hemodynamics
Recent MI and CABG candidates (6 pts) [49]	Autologous BM AC133+ cells	Intracardiac injection into infarct border zone	Improved LV function in four patients; improved infarct tissue perfusion in five patients.
Severe chronic ischemic heart failure (21 pts) [50]	Autologous BM-MNCs	Transendocardial delivery of 25×10^6 cells using electromechanical mapping system	Reduced infarcted area; improved cardiac hemodynamics at 4 months
Severe ischemic heart failure (8 pts) [51]	Autologous BM-MNCs	Transendocardial delivery using electromechanical mapping system	Improved myocardial perfusion and function
No-option patient with advanced coronary artery disease (10 pts) [52]	Autologous BM cells	Transendocardial delivery using electromechanical mapping system	Improved angina score and stress-induced ischemia
AMI with stent implantation (27 pts) [53]	PB stem cell (CD34 +) mobilized with G-CSF	Intracoronary infusion of 7×10^6 cells	Improved exercise capacity, myocardial perfusion, and systolic function, higher rate of in-stent restenosis
AMI with successful PCI-BOOST trial (60 pts) [54]	Autologous BM cells (30 pts), no cell (30 pts)	Intracoronary infusion	Enhanced LV systolic function, no adverse clinical events; no increase in in-stent restenosis; at 18 mos no significant effects on LV systolic function
AMI (TOPCARE) [33, 55]	Autologous BM cells, circulating progenitors	Intracoronary infusion	Enhanced LV systolic function
TOPCARE-CHD (75 pts) [59]	Autologous BM cells, circulating progenitors	Intracoronary infusion	Enhanced LV systolic function
REPAIR-AMI (204 pts) [60]	Autologous BM cells	Intracoronary infusion	Enhanced LV systolic function and fewer clinical events compared to control
ASTAMI (100 pts) [61]	Autologous BM cells	Intracoronary infusion	No change in LV function compared to control
Chronic MI (18 pts) [56]	Autologous BM stem cells given up to 8 yrs after MI	Intracoronary infusion	Improvement in global LV function, reduction of infarct size, infarction wall movement velocity, and oxygen uptake

AMI = acute MI; CABG = coronary artery bypass grafting; PB = peripheral blood; PCI = percutaneous coronary intervention; PTCA = percutaneous transluminal coronary angioplasty; pts = patients; LV = left ventricular; BM-MNCs = bone marrow mononuclear cells.

receive intracoronary infusion of BM cells or no treatment (no recatheterization, no placebo). The authors reported an increase in ejection fraction and a reduction of end systolic volumes in the group of patients receiving BM cells. Recently these authors reported on the long-term follow-up of these patients, revealing that the early benefit that had once been observed was not preserved [57]. The reasons for the apparent loss of benefit have not yet been resolved [58]. Other clinical trials using autologous BM-derived cells have shown a similar safety record and suggestions of functional improvement (Table 12.2) [59, 60]. A smaller placebo-controlled study did not reveal any improvement in systolic function [61].

Finally, a recent report provided early, uncontrolled evidence that LV function and exercise capacity increased, while the area of heart muscle damage shrank, in 18 patients given infusions of their own BM stem cells up to 8 years after a heart attack [56]. The results included an observed improvement in global LV function by 15%, an increase in infarct wall movement velocity, and a significant reduction of infarct size by 30%. These interesting observations, that await corroboration by randomized controlled trials, suggest that salvage of myocardial function may be possible very late after MI, in contrast to long-held paradigms [62].

Conclusion, limitations, and future perspectives

Despite incomplete evidence regarding the mechanisms of action, plasticity, transdifferentiation, etc. the demonstration of improvement in myocardial function induced following transplantation of autologous BM-derived stem/progenitor cells both in preclinical as well as in available clinical trials remains a potent force driving discovery and clinical development simultaneously and has provided new hope for patients with debilitating heart diseases.

Potential limitations of using autologous BM/peripheral blood-derived stem/progenitor cells have been identified and therefore must be acknowledged. Risk factors for coronary artery disease are reported to be associated with a reduced number and functional activity of EPCs in the peripheral blood of patients [63–66]. Likewise, patients with diabetes showed lower EPC numbers [67]. Similarly, in diabetic mice, EPC-mediated reendothelialization was impaired [68]. Whether these noted defects in EPCs from patients with preexisting risk factors or systemic diseases like diabetes also translate to other BM-derived stem cells has not been studied and would need to be investigated, although the notion of modifying EPC phenotype in order to overcome differences in potency has already been successfully tested [69, 70].

MI is primarily a disease of older persons, and the senescent myocardium may differ biologically from the myocardium of young persons and from that of the small adult animals typically used in initial stem cell experiments. In particular, the cells (myocytes) themselves and the intercellular messaging milieu in the interstitial space may be profoundly different under each clinical condition. Senescence in the aging myocardium has been characterized by the predominance of large myofibers expressing p19INK4—a marker of cellular aging and apoptosis [71]. It is likely that the molecular signals produced by such cells and their extracellular environment are not as favorable for stem cell differentiation, migration, and integration as are the signals present in younger hearts. These conditions need to be further characterized, as this knowledge may allow physicians to modify the environment, making it more conducive to successful stem cell treatment.

Many interesting questions remain unanswered. What population of adult BM-derived cells is most potent? How do BM-derived cells maintain themselves? How is their fate determined? Can BM-derived cells move from one tissue to another to provide regenerative cells? These questions quickly give rise to other questions centered around whether we can manipulate stem cells: can they be isolated for study or do they only maintain their stem cell abilities in their *in vivo* niches? Can they be culture expanded or does this change their identity, phenotype, and functional abilities? Can they only be delivered from exogenous sources to repair myocardium, or can their numbers be boosted *in situ*? How may they be better used to treat damaged, diseased, and senescent hearts? Could dedifferentiation of autologous, somatic, easily accessible cells be possible and could it be investigated for additional sources of functional multipotent, yet autologous cells? Some or many of these questions will

be answered in the near future, thereby paving new ways and insights regarding the therapeutic use of autologous human BM- derived cells for regeneration and repair of diseased myocardium. Until then, let us celebrate the breakthroughs and continue to refine our approaches for stem cell therapy.

References

1 Pfeffer MA. Left ventricular remodeling after acute myocardial infarction. Annu Rev Med 1995; 46:455–466.

2 McCulloch EA, Till JE. The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res 1960; 13:115–125.

3 Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275(5302):964–967.

4 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114(6):763–776.

5 Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103(5):634–637.

6 Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. N Engl J Med 2002; 346(1):5–15.

7 Kawamoto A, Tkebuchava T, Yamaguchi J, et al. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107(3):461–468.

8 Yoon YS, Wecker A, Heyd L, et al. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 2005; 115(2):326–338.

9 Iwasaki H, Kawamoto A, Ishikawa M, et al. Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 2006; 113(10):1311–1325.

10 Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease: part II: cell-based therapies. Circulation 2004; 109(22):2692–2697.

11 Muller P, Pfeiffer P, Koglin J, et al. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. Circulation 2002; 106(1):31–35.

12 Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res 2002; 90(6):634–640.

13 Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001; 7(4):430–436.

14 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 401:701–705.

15 Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001; 98(18):10344–10349.

16 Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003; 107(7):1024–1032.

17 Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 2005; 115(3):572–583.

18 Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107(11):1395–1402.

19 Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 2004; 95(1):9–20.

20 Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418(6893):41–49.

21 Shyu KG, Wang BW, Hung HF, et al. Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. J Biomed Sci 2006; 13(1):47–58.

22 Liechty KW, MacKenzie TC, Shaaban AF, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med 2000; 6(11):1282–1286.

23 Fernandez-Aviles F, San Roman JA, Garcia-Frade J, et al. Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. Circ Res 2004; 95(7):742–748.

24 Yeh ET, Zhang S. A novel approach to studying transformation of human stem cells into cardiac cells in vivo. Can J Cardiol 2006; 22 (suppl B):66B–71B.

25 Ma N, Stamm C, Kaminski A, et al. Human cord blood cells induce angiogenesis following myocardial infarction in NOD/SCID-mice. Cardiovasc Res 2005; 66(1):45–54.

26 Zhang S, Wang D, Estrov Z, et al. Both cell fusion and transdifferentiation account for the transformation of human peripheral blood CD34-positive cells into cardiomyocytes in vivo. Circulation 2004; 110(25):3803–3807.

27 Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002; 105(1):93–98.

28 Yeh ET, Zhang S, Wu HD, et al. Transdifferentiation of human peripheral blood CD34+-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. Circulation 2003; 108(17):2070–2073.

29 Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 2000; 97(7):3422–3427.

30 Urbich C, Heeschen C, Aicher A, et al. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 2003; 108(20):2511–2516.

31 Ito H, Maruyama A, Iwakura K, et al. Clinical implications of the "no reflow" phenomenon. A predictor of complications and left ventricular remodeling in reperfused anterior wall myocardial infarction. Circulation 1996; 93(2):223–228.

32 Gibson CM, Cannon CP, Murphy SA, et al. Relationship of the TIMI myocardial perfusion grades, flow grades, frame count, and percutaneous coronary intervention to long-term outcomes after thrombolytic administration in acute myocardial infarction. Circulation 2002; 105(16):1909–1913.

33 Assmus B, Schachinger V, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002; 106(24):3009–3017.

34 Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003; 9(9):1195–1201.

35 Balsam LB, Wagers AJ, Christensen JL, et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004; 428(6983):668–673.

36 Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428(6983):664–668.

37 Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411):143–147.

38 Shi Q, Rafii S, Wu MH-D, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998; 92:362–367.

39 Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85(3):221–228.

40 Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999; 5(4):434–438.

41 Iwakura A, Luedemann C, Shastry S, et al. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation 2003; 108(25):3115–3121.

42 Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003; 362(9385):697–703.

43 Terada N, Hamazaki T, Oka M, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002; 416(6880):542–545.

44 Ying QL, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. Nature 2002; 416(6880):545–548.

45 Koyanagi M, Brandes RP, Haendeler J, et al. Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: a novel mechanism for cell fate changes? Circ Res 2005; 96(10):1039–1041.

46 Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 2003; 425(6961):968–973.

47 Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001; 104(9):1046–1052.

48 Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002; 106(15):1913–1918.

49 Stamm C, Westphal B, Kleine HD, et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003; 361(9351):45–46.

50 Perin EC, Dohmann HF, Borojevic R, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 2003; 107(18):2294–2302.

51 Tse HF, Kwong YL, Chan JK, et al. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet 2003; 361(9351):47–49.

52 Fuchs S, Satler LF, Kornowski R, et al. Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility study. J Am Coll Cardiol 2003; 41(10):1721–1724.

53 Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004; 363(9411):751–756.

54 Wollert KC, Meyer GP, Lotz J, et al. Randomized controlled clinical trial of intracoronary autologous bone marrow cell transfer post myocardial infarction [Abstract]. Circulation 2003; 108:2723.

55 Britten MB, Abolmaali ND, Assmus B, et al. Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. Circulation 2003; 108(18):2212–2218.

56 Strauer BE, Brehm M, Zeus T, et al. Regeneration of human infarcted heart muscle by intracoronary autologous bone marrow cell transplantation in chronic coronary artery disease: the IACT study. J Am Coll Cardiol 2005; 46(9):1651–1658.

57 Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (bone marrow transfer to enhance ST-elevation infarct regeneration) trial. Circulation 2006; 113(10):1287–1294.

58 Welt FG, Losordo DW. Cell therapy for acute myocardial infarction: curb your enthusiasm? Circulation 2006; 113(10):1272–1274.

59 Assmus B, Honold J, Schachinger V, et al: Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl J Med 2006; 355:1222–1232.

60 Schachinger V, Erbs S, Elsasser A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006; 355:1210–1221.

61 Lunde K, Solheim S, Aakhus V, et al: Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 2006; 355:1199–1209.

62 Reimer KA, Lowe JE, Rasmussen MM, Jennings RB. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. Circulation 1977; 56(5):786–794.

63 Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001; 103 :2885–2890.

64 Walter DH, Haendeler J, Reinhold J, et al. Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res 2005; 97(11):1142–1151.

65 Schmidt-Lucke C, Rossig L, Fichtlscherer S, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 2005; 111(22):2981–2987.

66 Urbich C, Dimmeler S. Risk factors for coronary artery disease, circulating endothelial progenitor cells, and the role of HMG-CoA reductase inhibitors. Kidney Int 2005; 67(5):1672–1676.

67 Tepper OM, Galiano RD, Capla JM, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002; 106(22):2781–2786.

68 Ii M, Takenaka H, Asai J, et al. Endothelial progenitor thrombospondin-1 mediates diabetes-induced delay in reendothelialization following arterial injury. Circ Res 2006; 98(5):697–704.

69 Iwaguro H, Yamaguchi J, Kalka C, et al. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 2002; 105(6):732–738.

70 Murasawa S, Llevadot J, Silver M, et al. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. Circulation 2002; 106(9):1133–1139.

71 Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ Res 2003; 92(2):139–150.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

13 CHAPTER 13

Endothelial progenitor cells and the infarcted heart

Stefanie Dimmeler, PhD, *Masamichi Koyanagi,* MD, *Carmen Urbich,* PhD, *& Andreas M. Zeiher,* MD

Introduction

Despite the development of novel therapeutic strategies, electrophysiological and surgical treatment regimens, heart failure remains one of the major causes of mortality in the Western world. Heart failure can develop as a consequence of myocardial infarction despite coronary revascularization procedures. Ischemia-induced death of cardiomyocytes results in scar formation and reduced contractility of the ventricle. Various experimental studies provide evidence that the infusion or injection of stem/progenitor cells after myocardial infarction reduces scar formation and fibrosis and preserves cardiac function. Moreover, different subsets of progenitor cells have been shown to augment myocardial blood flow. Both effects may be related to a direct physical effect (differentiation of progenitor cells to endothelial cells, smooth muscle cells, and cardiomyocytes) and/or the release of paracrine factors by progenitor cells, which prevent apoptosis of cardiomyocytes or modulate scar development (Figure 13.1). This chapter will focus on the role of endothelial progenitor cells (EPCs) in cardiovascular repair.

Characterization of EPCs

The existence of circulating cells, which can differentiate to the endothelial lineage *in vitro* and *in vivo*, was first described by Asahara et al. in 1997 [1]. This study demonstrated that purified $CD34^+$ hematopoietic progenitor cells from adults can differentiate *ex vivo* to an endothelial phenotype (i.e., EPCs). A similar finding was reported in 1998 [2]. Again, a subset of $CD34^+$ hematopoietic stem cells was shown to differentiate to the endothelial lineage and to express endothelial marker proteins such as vWF and incorporated Di-LDL (Dil-acetylated LDL). Moreover, bone marrow cell (BMC)-transplanted genetically tagged cells were covering implanted Dacron grafts [2]. On the basis of these pioneering studies and the assumption that these cells may represent the circulating hemangioblasts in the adult, EPC were characterized by the coexpression of hematopoietic stem cell markers such as CD34 or CD133 and endothelial marker proteins like the vascular endothelial growth factors receptor-2 (VEGFR2/KDR). Although this concept is still accepted by the scientific community, increasing evidence suggests that related cell types may also play a role in vascular repair. A subset of myeloid cells also has the capacity to acquire an endothelial phenotype. $CD14^+/CD34^-$ myeloid cells [3] or purified $CD14^+$ mononuclear cells can coexpress endothelial markers, form tube-like structures *ex vivo*, and incorporate in newly formed blood vessels *in vivo* [4]. Two recent studies identified two subpopulations within the $CD14^+$ cells, which are characterized by a low CD34 expression [5] or the coexpression of KDR [6], with a high plasticity and endothelial repair capacity. These data would suggest that there are at least two subpopulations of cells in the peripheral blood, which have the capacity to differentiate into endothelial cells: myeloid subpopulations and cells expressing

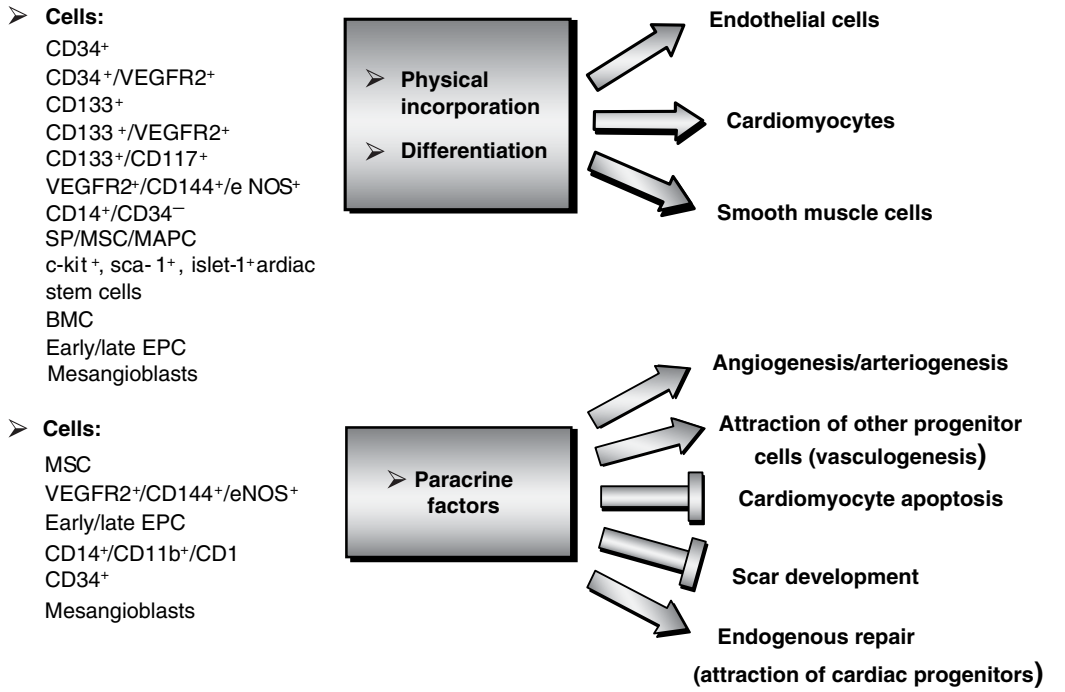


Figure 13.1 Function of progenitor cell populations. The upper panel summarizes the progenitor cell populations which differentiate to lineages of the endothelial cells, smooth muscle cells, and/or cardiomyocytes. The lower panel summarizes the progenitor cell populations which exert a paracrine effect.

hematopoietic stem cell markers likely resembling the "hemangioblast." It is possible that these two subpopulations reflect the different cells appearing in culture assays, the "early EPC" and the "late outgrowing EPC," respectively [7]. It is being discussed at present whether the myeloid cells or early EPCs are indeed "progenitor cells" or whether these cells are functionally active proangiogenic and repair cells without a real "progenitor" characteristic. Interestingly, lineage tracking showed that myeloid cells are the hematopoietic stem-cell-derived intermediates, which contribute to muscle regeneration. A subset of human peripheral blood monocytes additionally was proposed to act as pluripotent stem cells, indicating that at least a subpopulation within these cells may indeed exert a progenitor cell function.

Notably, several studies demonstrated that other cell populations beside hematopoietic stem cells and myeloid cells can give rise to endothelial cells. Adult BM-derived stem/progenitor cells (e.g., the "side population" cells), mesenchymal stem cells, and multipotent adult progenitor cells, which are distinct from hematopoietic stem cells, have also been shown to differentiate to the endothelial lineage [8, 9]. In addition, non-BM-derived cells, such as tissue-resident cardiac stem cells, are capable of differentiating to the endothelial lineage [10]. These data would suggest that the adult organism is equipped with a set of different cells responsible for endothelial and vascular repair. Future studies will have to elucidate the physiologically most important EPC population and, in addition, the cells best suited for cell therapy of ischemic diseases.

Functional capacity of EPCs

Experimental studies

Improving neovascularization after critical ischemia is a major therapeutic goal in cardiovascular medicine. On the basis of the discovery of circulating and BM-derived EPCs, multiple studies have tested the capacity of different cell populations

to augment neovascularization, and thereby increase the blood supply to ischemic tissue. In animal models of hind limb ischemia or myocardial infarction, the injection of various subsets of cells significantly improved blood flow and cardiac function and reduced left ventricular scarring (for review, see [11]). The progenitor cells used include isolated human $CD34^+$ or $CD133^+$ cells, G-CSF mobilized $CD117^+/CD34^+$ cells [12], and *ex vivo* cultivated peripheral blood-derived EPCs [13]. It is noteworthy that mature endothelial cells did not exert a beneficial effect in various studies [12, 14]. Moreover, the infusion of total $CD14^+$ did not augment neovascularization without enrichment of the EPC subpopulations within this cell population [4].

Clinical trials

The results of initial pilot trials in humans indicate that BM-derived or circulating blood-derived progenitor cells are useful for therapeutically improving blood supply to ischemic tissue. As summarized in Tables 13.1 and 13.2, the published studies demonstrate that the infusion of autologous BMC is both safe and feasible in patients with myocardial infarction [15–24]. The studies by Strauer et al. [15], TOPCARE-AMI [16], BOOST [17], and Fernandez-Aviles et al. [18] documented an improvement in left ventricular ejection fraction. A recent randomized, controlled trial by Janssens et al. [19] did not reveal a significant effect on global ejection fraction; however, it showed an increase in regional ejection fraction and a reduction of infarct size in the BMC-treated group. Only one study, the ASTAMI trial, did not show any benefit [20]. The beneficial effects seen in most of the pilot studies were confirmed by findings from the recent multicenter REPAIR-AMI trial [21]. The study results demonstrated a significant improvement of global and regional ejection fraction in the BMC-treated group. Notably, all of the cited studies used the total population of BM mononuclear cells, which contain roughly 2–3% $CD34^+$ cells and 1% EPCs. Given that the BMCs also contain side population cells, mesenchymal cells, and potential other not yet identified cell populations, the specific contribution of EPCs is unclear. However, a few studies did use *ex vivo* expanded circulating blood-derived EPCs. In one group of the TOPCARE-AMI trial, *ex vivo* cultivated EPCs were infused in patients with myocardial infarction. This study demonstrated a similar effect of EPC compared to BMC [16]. Moreover, Erbs et al. showed that granulocyte colony-stimulating factor-mobilized *ex vivo* cultivated EPCs improved the ejection fraction in patients with chronic ischemic heart disease [25], suggesting that EPCs, which can be easily isolated from the peripheral blood without BM aspiration, might be used as an alternative for BMCs. However, in contrast to the study in acute myocardial infarction patients, *ex vivo* cultivated EPCs—isolated from nonmobilized patients with chronic ischemic postinfarction heart failure—were significantly less effective compared to BMCs in a recently published study [26]. This might be explained by the lower number of EPC (22 Mio compared to 205 Mio BMC) or a different functionality (e.g., differentiation capacity, homing) of EPC in chronic versus acute patients.

Overall, the available clinical data indicate that cell therapy with EPCs or BMCs is both safe and feasible and is associated with improved heart function. A recent study questioned whether the improvement seen during 6 months is maintained over time [27]. However, careful evaluation of the 18 months follow-up data of the BOOST trial indicates that the ejection fraction of the cell therapy group is maintained from 6 to 18 months follow-up. The lack of a statistically significant difference between the groups at 18 months is caused by a catch-up of the control group. Given the small number of patients (30 per group), it is not surprising to see no statistical difference between the two groups. The long-term 2 year follow-up data of the TOPCARE-AMI trial clearly demonstrate that the ejection fraction is maintained and even further augmented in BMC- or EPC-treated patients (S. Dimmeler and A.M. Zeiher, unpublished data).

Mechanism of action

EPCs are incorporated to a significant extent into the capillaries after ischemia, and thereby physically contribute to the formation of new capillaries in the ischemic tissue. However, the varying numbers of incorporated cells with an endothelial phenotype may not entirely explain the strong therapeutic effect observed after cell therapy in experimental

Table 13.1 Safety and feasibility studies using stem cells for myocardial infarction.

Study	*n*	*Days post-AMI*	*Cell type*	*Cell preparation*	*Cell number ($\times 10^6$)*	*Safety*	*Myocardial function*
Strauer et al. [15]	10 11/03: 40 vs. control	8	BMC	40 mL, Ficoll—overnight teflon	28	+	Rregional contractility ⇑ (LVA) Endsystolic volume ⇓ (LVA) Perfusion ⇑ (Scintigraphy)
TOPCARE–AMI [16]	59	4.9	CPC	250 mL/3-day culture	*16*	+	Global contractility ⇑ (LVA/MRI) Endsystolic volume ⇓ (LVA/MRI)
			BMC	50 mL/Ficoll—same day	213		Viability ⇑ (MRI) Flow reserve ⇑ (Doppler)
Fernandez-Aviles [18]	20	13.5	BMC	50 mL, Ficoll—overnight teflon	78	+	Global contractility ⇑ (MRI) Endsystolic volume ⇓ (MRI)
Ruan et al. [22]	10 vs. 10 (rand.)	0	BMC vs. saline			+	Global contractility ⇑ (LVA)
Mansour et al. [23]	12	14	$CD133^+$ BMC	Mouse antibody		Stenosis?	Global contractility ⇑ (LVA)
Chen et al. [24]	34 vs. 35	18	B-MSC vs. control	10-day culture		+	Global contractility ⇑ (LVA)

B-MSC = bone-marrow-derived mesenchymal stem cells; LVA = left ventricular angiography; rand. = randomized; CPC = cardiac progenitor cells; MRI = magnetic resonance imaging.

Table 13.2 Randomized trials

Study	*n*	*Days post-AMI*	*Cell type*	*Cell preparation*	*Cell number ($\times 10^6$)*	*Safety*	*Myocardial function*
Single/two center(s)							
BOOST [17, 22]	60 1:1 rand.	4.8	BMC vs. rand. control	Gelatine polysuccinate – same day infusion	2460	+	6 months: global contractility ⇑ (MRI) 18 months: no significant difference (MRI) 18 months: diastolic dysfunction ⇓ (Echo)
Janssens et al. [19]	67 1:1 rand.	<24h	BMC vs. i.c. placebo	Ficoll density gradient centrifugation – few hours after acute PCI	304	+	Global contractility no change Infarct size ⇓ (MRI)
ASTAMI [20]	100 1:1 rand.	6	BMC vs. rand. control	Lymphoprep™ – next day infusion	87	+	Global contractility no change
Multicenter—placebo controlled, double-blind							
REPAIR–AMI [21]	204 1:1 rand.	4	BMC vs. i.c. placebo	Ficoll density gradient centrifugation – same or next day infusion	234	+	Global contractility ⇑ (QLVA)

rand. = randomized; Echo = Echocardiography; QLVA = quantitative left ventricular angiography; BMC = bone marrow cells; MRI = magnetic resonance imaging; PCI = percutaneous coronary intervention.

models. Thus, the efficiency of EPC-induced neovascularization may not solely be due to the incorporation of EPCs into newly formed vessels, but may also be influenced by the release of proangiogenic factors in a paracrine manner. In line with this hypothesis, the deletion of Tie-2-$^+$ BM-derived cells through activation of a suicide gene blocked tumor angiogenesis, even though in this study cells were not found to be integrated into tumor vessels but rather were located adjacent to the vessel wall [28]. Moreover, recent studies suggest that monocytic cells as well as mesenchymal stem cells augment arteriogenesis via the release of angiogenic growth factors [29].

In addition, circulating blood-derived EPCs were shown to acquire a cardiomyogenic phenotype. In a coculture system, coincubation of human EPCs with neonatal cardiomyocytes triggered the expression of cardiac genes in the human cells as assessed by RT-PCR, immunostaining, and FACS analysis [30–32]. In this coculture assay, gap-junctional communication between neonatal cardiomyocytes and differentiating stem/progenitor cells was detected [30]. Functional activity of the differentiated circulating progenitor cells was further documented by showing oscillating calcium transients after pacing [30]. The *in vitro* differentiation of EPCs toward the cardiac lineage was additionally confirmed *in vivo* after injection of *ex vivo* expanded circulating blood-derived EPCs [33] or isolated CD34$^+$ cells, which are enriched for EPCs [34], in an experimental myocardial infarction model. In contrast, a recent study was unable to detect differentiation of *ex vivo* expanded EPCs in a coculture model [35]. The reason for this discrepancy may be explained by the low percentage of cardiomyocytes in the coculture system used by the authors. As shown in Figure 13.2C, differentiation of EPC was detected when the number of cardiomyocytes exceeds more than 50% at the end of the coculture experiments.

Cell fusion has been raised as an alternative mechanism underlying the acquirement of a cardiac phenotype by progenitor cells. To assess the incidence of cell fusion, we incubated DiLDL-labeled EPCs with GFP (green fluorescence protein)-transfected cardiomyocytes. Indeed, already after 2 days of coculture, about 2% double-positive cells were detectable. However, careful analysis of nuclear fusion excluded a significant contribution of the classical fusion events [32, 33]. Further experiments indicate that the transfer of proteins between EPCs and cardiomyocytes was predominantly due to the formation of nanotubular structures between cells, allowing the transport of proteins and even larger organelles such as mitochondria [36]. Also, a recent study reported that the transport of mitochondria from mesenchymal stem cells to mitochondria-deficient cells rescued aerobic respiration [37]. Although the *in vivo* relevance of these *in vitro* experimental findings so far is unclear, the transport of proteins or organelles may affect the cell fate and function of progenitor cells as well as the connected cardiomyocytes.

Potential limitations of EPC-mediated repair

One potential limitation for the use of autologous EPCs is the documented impairment of both EPC number and function in patients with coronary artery disease and heart failure. The number of circulating EPCs and the functional capacity indicated by the migration toward chemokines is significantly down-regulated in patients with coronary artery disease [38]. Likewise, the number of cultured EPCs out of peripheral blood mononuclear cells from patients with diabetes was significantly reduced [39]. Severe heart failure patients also showed a marked reduction of EPC number and function [40].

A second limitation might be the efficiency of incorporation and regeneration capacity of EPCs in clinical trials. Given the rather small number of infused cells (20–200 Mio) and the fact that maximally 10% of the cells accumulate in the heart (S. Dimmeler and A.M. Zeiher, unpublished data), it is unclear how the millions of cardiomyocytes, which are lost during a severe infarct, can be replaced or compensated. The infused cells of course may exert paracrine effects as discussed above. However, future strategies in myocardial regeneration will need to augment cell homing and improve the regeneration of the myocardium in its pure sense by generation of new cardiomyocytes. Whether this approach to regeneration will require the use of other cell sources or novel strategies to force homing and differentiation by cell pretreatment (e.g., with growth factors or small molecules) needs to be assessed.

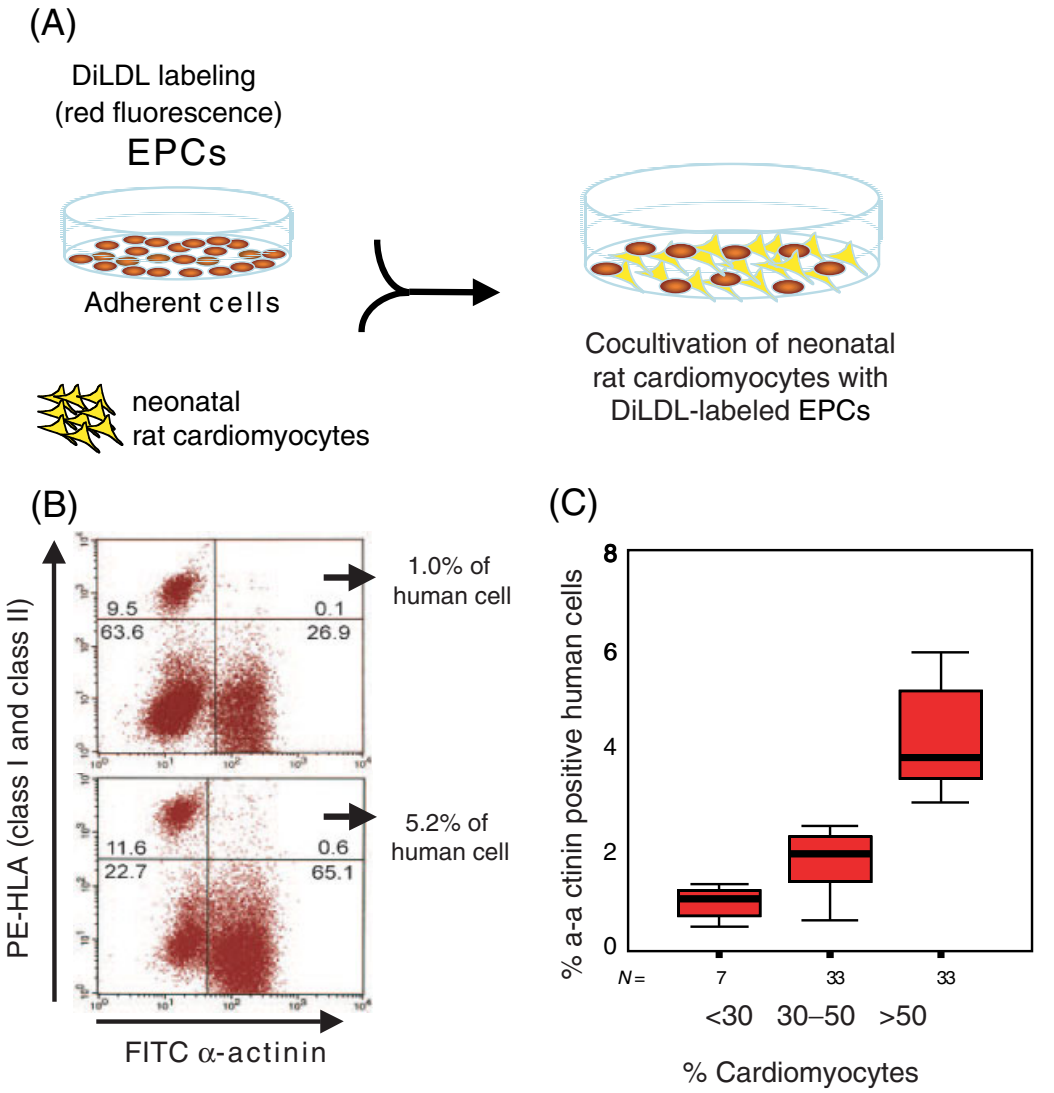


Figure 13.2 Cardiac differentiation of EPCs *in vitro*. Illustration of the experimental setup of the coculture system. (B) Representative FACS analysis of EPC differentiation in a coculture with low percentage of cardiomyocytes (upper panel) or high concentration of cardiomyocytes (lower panel). (C) Quantification of cardiac differentiation of EPCs in relation to the number of cardiomyocytes in the coculture. PE-HLA = phycoerythrin-human leukocyte antigen; FITC = fluorescein isothiocyanate.

Acknowledgment

The authors apologize for the failure to cite many important studies in the field because of space limitations. The present studies are supported by the Deutsche Forschungsgemeinschaft, the Alfried Krupp Foundation, and the Leducq Foundation.

References

1 Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275:964–967.

2 Shi Q, Rafii S, Wu MH, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998; 92:362–367.

3 Schmeisser A, Garlichs CD, Zhang H, et al. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res 2001; 49:671–680.

4 Urbich C, Heeschen C, Aicher A, et al. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 2003; 108:2511–2516.

5 Romagnani P, Annunziato F, Liotta F, et al. CD14+CD34 low cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors. Circ Res 2005; 97(4): 314–322.

6 Elsheikh E, Uzunel M, He Z, et al. Only a specific subset of human peripheral blood monocytes has endothelial-like functional capacity. Blood 2005; 106(7):2347–2355.

7 Hur J, Yoon CH, Kim HS, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004; 24:288–293.

8 Reyes M, Dudek A, Jahagirdar B, et al. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002; 109:337–346.

9 Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107:1395–1402.

10 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.

11 Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 2004; 95:343–353.

12 Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001; 7:430–436.

13 Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103:634–637.

14 Urbich C, Heeschen C, Aicher A, et al. Cathepsin L is required for endothelial progenitor cell-induced neovascularization. Nat Med 2005; 11:206–213.

15 Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002; 106:1913–1918.

16 Assmus B, Schachinger V, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002; 106:3009–3017.

17 Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 2004; 364:141–148.

18 Fernandez-Aviles F, San Roman JA, Garcia-Frade J, et al. Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. Circ Res 2004; 95:742–748.

19 Janssens S, Dubois C, Bogaert J, et al. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet 2006; 367:113–121.

20 Lunde K, Solheim S, Aakhus V, et al: Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 2006; 355:1199–1209.

21 Schachinger V, Erbs S, Elsasser A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355, 1210–1221, 2006.

22 Ruan W, Pan CZ, Huang GQ, et al. Assessment of left ventricular segmental function after autologous bone marrow stem cells transplantation in patients with acute myocardial infarction by tissue tracking and strain imaging. Chin Med J (Engl) 118, 1175–1181, 2005.

23 Mansour S, Vanderheyden M, De Bruyne B, et al. Intracoronary delivery of hematopoietic bone marrow stem cells and luminal loss of the infarct-related artery in patients with recent myocardial infarction. J Am Coll Cardiol 47, 1727–1730, 2006.

24 Chen SL, Fang WW, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 94, 92–95, 2004.

25 Erbs S, Linke A, Schuler G, Hambrecht R. Intracoronary administration of circulating blood-derived progenitor cells after recanalization of chronic coronary artery occlusion improves endothelial function. Circ Res 2006; 98:e48.

26 Assmus B, Honold J, Schachinger V, et al. Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl Med 2006;355(12):1222–1232.

27 Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (bone marrow transfer to enhance ST-elevation infarct regeneration) trial. Circulation 2006; 113:1287–1294.

28 De Palma M, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 2003; 9:789–795.

29 Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003; 107:1164–1169.

30 Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003; 107:1024–1032.

31 Koyanagi M, Haendeler J, Badorff C, et al. Non-canonical Wnt signaling enhances differentiation of human circulating progenitor cells to cardiomyogenic cells. J Biol Chem 2005; 280:16838–16842.

32 Koyanagi M, Urbich C, Chavakis E, et al. Differentiation of circulating endothelial progenitor cells to a cardiomyogenic phenotype depends on E-cadherin. FEBS Lett 2005; 579:6060–6066.

33 Murasawa S, Kawamoto A, Horii M, et al. Niche-dependent translineage commitment of endothelial progenitor cells, not cell fusion in general, into myocardial lineage cells. Arterioscler Thromb Vasc Biol 2005; 25: 1388–1394.

34 Iwasaki H, Kawamoto A, Ishikawa M, et al. Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 2006; 113:1311–1325.

35 Gruh I, Beilner J, Blomer U, et al. No evidence of transdifferentiation of human endothelial progenitor cells into cardiomyocytes after coculture with neonatal rat cardiomyocytes. Circulation 2006; 113:1326–1334.

36 Koyanagi M, Brandes RP, Haendeler J, et al. Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: a novel mechanism for cell fate changes? Circ Res 2005; 96:1039–1041.

37 Spees JL, Olson SD, Whitney MJ, Prockop DJ. Mitochondrial transfer between cells can rescue aerobic respiration. Proc Natl Acad Sci USA 2006; 103:1283–1288.

38 Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001; 89:E1–E7.

39 Tepper OM, Galiano RD, Capla JM, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002; 106:2781–2786.

40 Valgimigli M, Rigolin GM, Fucili A, et al. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation 2004; 110:1209–1212.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

14 CHAPTER 14

Cytokines and heart remodeling

Hiroyuki Takano, MD, PhD, *& Issei Komuro*, MD, PhD

Introduction

Since the clinical outcome after an acute myocardial infarction (AMI) depends on the extent of damaged myocardium, myocardial salvage by reperfusion therapies such as thrombolysis and percutaneous transluminal coronary intervention (PCI) has become very important in the treatment of patients with AMI. Although the use of these approaches has reduced mortality from AMI, these therapies have also led to a dramatic increase in the number of surviving patients suffering from heart failure [1, 2]. Recently, medical interest in myocardial regeneration has grown and research on gene- and cell-based therapies has progressed. The use of bone marrow stem cells (BMSCs) has been evaluated as a potential tool in regenerative medicine. Several hematopoietic cytokines, including interleukin (IL)-3, granulocyte–macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor, stem cell factor (SCF), and erythropoietin, have been known to regulate the growth and differentiation of hematopoietic progenitor cells. Additionally, these cytokines have been shown to mobilize BMSCs [3]. It has been recently demonstrated that BMSCs differentiate into cardiomyocytes, endothelial cells, and vascular smooth muscle cells in the mouse model of AMI [4]. In addition, cytokine-mediated recruitment of BMSCs has been reported to improve cardiac dysfunction and reduce mortality after AMI in mice [5]. In this chapter, we discuss how G-CSF can prevent left ventricular (LV) remodeling and dysfunction after AMI in various animal models [6–11].

Myocardial infarction and cardiac remodeling

Therapeutic advances have improved the survival of patients with AMI. However, the improved survival of patients with myocardial infarction (MI) has led to an increase in the number of patients suffering from heart failure [1, 2]. Congestive heart failure (CHF), which is mostly caused by MI, remains one of the major causes of mortality in the Western world. Despite the development of pharmacological and mechanical revascularization techniques, heart failure proceeds as a consequence of MI. Therefore, more effective treatment options for patients with AMI need to be developed.

The term LV remodeling was previously described as a physiologic and pathologic condition that occurs after AMI (see also Chap. 21). The process of LV remodeling begins with cardiomyocyte loss due to necrosis or apoptosis and continues with cardiomyocyte lengthening, LV wall thinning, infarct expansion, LV dilation, cardiomyocyte hypertrophy, and collagen accumulation [2]. Complex architectural alterations are induced in both infarcted and noninfarcted myocardium after AMI. Dilatation of the LV and infarcted wall thinning are the prominent features in the infarcted region. In addition, LV remodeling with compensatory dilation and hypertrophy is induced in the noninfarcted region. Cardiac remodeling is also recognized as a process occurring in other cardiovascular diseases such as systemic hypertension, valvular heart disease, myocarditis, and dilated cardiomyopathy [12]. The process of cardiac remodeling is influenced by hemodynamic load, neurohumoral activation, and other factors [12]. Although cardiac

remodeling is initially an adaptive response to maintain normal function, it gradually becomes maladaptive and subsequently leads to progressive decompensation and CHF. The degree of post-MI remodeling roughly depends on infarct size. A large infarction induces greater dilation of the LV and more of an increase in wall stress of the LV than a small infarction. Although cardiomyocytes play critical roles in the remodeling process, cardiac fibroblasts, the coronary vasculature, and the extracellular matrix (ECM) are also involved in the process [1, 2, 12].

Many mediators, such as neurohumoral factors, cytokines, growth factors, and enzymes, are known to be involved in the progress of cardiac remodeling. There is accumulating evidence to suggest that neurohumoral factors such as angiotensin II (Ang II), aldosterone, endothelin-1, and norepinephrine play pivotal roles in the development of LV remodeling [1, 2, 12]. In particular, activation of the local renin–angiotensin system in myocardium has been found to be important. Many components of the renin–angiotensin system, including angiotensinogen, angiotensin-converting enzyme, and the Ang II type 1 receptor, are up-regulated in the heart after MI. Ang II induces hypertrophy of cardiomyocytes and increases proliferation of cardiac fibroblasts and collagen synthesis. Endothelin-1 is involved in the remodeling process that includes cardiac hypertrophy and collagen synthesis. Cytokines such as tumor necrosis factor-α and IL-6 also play important roles in cardiac remodeling. Cytokines are secreted in response to a variety of stimuli. Growth factors such as transforming growth factor (TGF)-β, fibroblast growth factor, and platelet-derived growth factor are shown to be associated with remodeling. TGF-β_1 plays an important role in the regulation of ECM production and fibrosis. Enzymes such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases have been recently shown to be involved in the remodeling process [1, 2, 12]. The degradation of ECM within the myocardium plays an important role in the progression of cardiac remodeling and heart failure after MI [13]. MMPs are a family of proteolytic enzymes for ECM degradation and are involved in tissue remodeling processes that include morphogenesis and wound healing (Figure 14.1).

Cardiomyocyte death, which is induced by necrosis or apoptosis, is an important cause of cardiac remodeling. Apoptosis, known as programmed cell death, is a fundamental physiologic and pathologic mechanism of cell death during life. Myocardial apoptosis peaks at 4–12 hours after AMI and is persistently detected up to 10 days in rats [14]. In the acute phase of MI, apoptosis as well as necrosis have been reported to represent the major form of cardiomyocyte death. Persistent apoptosis still occurs in the infarcted region even during the subacute phase (up to 60 days) in the human heart [14]. Moreover, a strong correlation between the apoptotic rate and the degree of LV remodeling was recognized in the phase, and this LV remodeling results in CHF.

Therefore, preventing the remodeling process would be an important therapeutic approach for heart failure after AMI. Therapeutic agents such as angiotensin-converting enzyme inhibitors and β-blockers are used to reduce cardiac remodeling and to decrease morbidity and mortality in patients with heart failure. Although cardiomyocytes are believed to be terminally differentiated cells without the ability to regenerate, it was recently demonstrated that cardiomyocytes proliferate after an MI in humans [15]. Therefore, there is the possibility that enhancement of the regeneration of cardiomyocytes, as well as stimulation of neovascularization, may prevent cardiac remodeling and the progression of heart failure. Since a reduction in the number of cardiomyocytes with pathologic conditions causes LV dilatation and hypertrophy of the remaining cardiomyocytes, myocardial regeneration appears to be a promising strategy to reduce adverse cardiac remodeling.

Cardioprotective effects of G-CSF on AMI

Orlic et al. examined whether cytokines could increase BMSC mobilization to the injured myocardium to promote myocardial regeneration [5]. In their study, mice were injected with rat SCF and recombinant human G-CSF once a day for 5 days before ligation of the left coronary artery was performed. SCF and G-CSF were given for 3 more days. Cytokine-mediated mobilization of BMSCs was found to result in myocardial regeneration

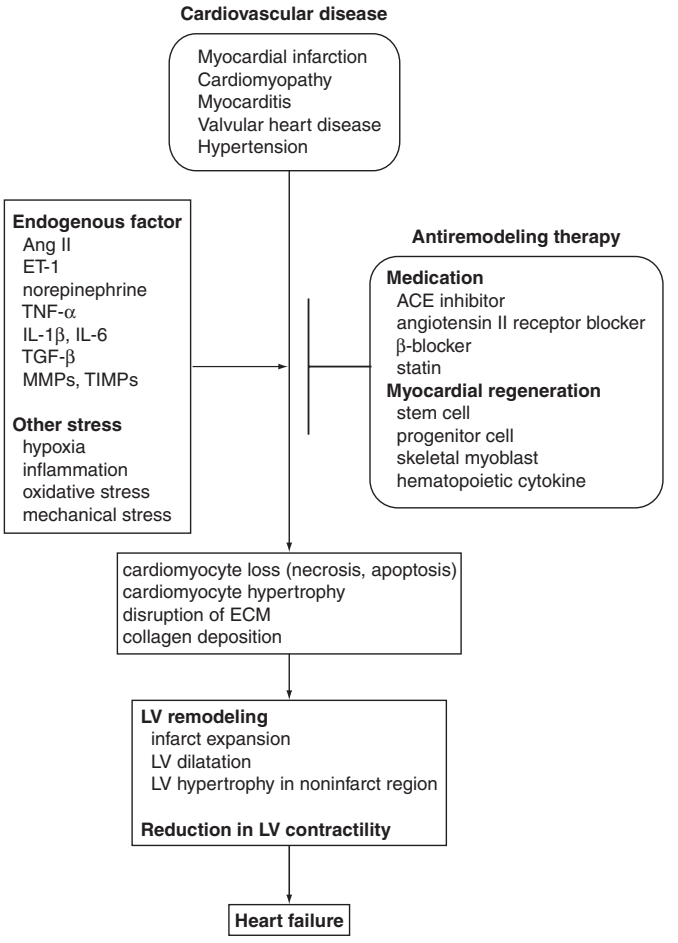


Figure 14.1 Cardiac remodeling. ACE = angiotensin-converting enzyme; Ang II = angiotensin II; ECM = extracellular matrix; ET-1 = endothelin-1; IL = interleukin; LV = left ventricle; MMPs = matrix metalloproteinases; TGF-β = transforming growth factor-β; TIMPs = tissue inhibitors of metalloproteinases; TNF-α = tumor necrosis factor-α.

characterized by dividing cardiomyocytes and formation of vascular structures 27 days after the AMI [5]. This preinfarction approach was found to significantly reduce mortality and improve cardiac function, such as ejection fraction, LV diameter, and LV pressure. However, the cytokine treatment was started before the MI, and a clinically relevant protocol involving post-MI administration of G-CSF alone was not examined in this study. Therefore, we examined whether or not a single treatment with G-CSF started after AMI could have beneficial effects [6]. In this study, mice underwent ligation of the left coronary artery and then were divided into four groups: (1) administration of vehicle (control group), (2) administration of G-CSF (100 μg/kg/day) and SCF (200 μg/kg/day) from 5 days before MI through 3 days after (pre-GS group), (3) administration of G-CSF (100 μg/kg/day) and SCF (200 μg/kg/day) for 5 days after MI (post-GS group), and (4) administration of G-CSF (100 μg/kg/day) alone for 5 days after MI (post-G group). In the post-GS and post-G groups, G-CSF or SCF was injected subcutaneously, 2 hours after MI. All three treatment groups with G-CSF showed less LV remodeling and improved cardiac function and survival rate after MI [6]. The number of apoptotic cells was decreased in the border area of all the treatment groups with G-CSF. We next used mice that had undergone replacement with BM cells with enhanced green fluorescent protein (GFP)-expression to elucidate the role of BM cells in the improvement of cardiac function. Many GFP-positive cells were recognized in the border area of all three treatment groups, but not the control

group. Most of the GFP-positive cells were infiltrated blood cells and some GFP-positive cells were observed at capillary walls [6]. There were few GFP-positive cardiomyocytes in the border area, as well as in the infarcted and remote areas. Therefore, we next compared the number of vessels in the border area. The number of capillaries in the border area after MI was much greater in all three treatment groups than in the control group. It was therefore concluded that if cytokine treatment is started after MI, it could reduce LV remodeling and dysfunction, at least in part, through an increase in neovascularization and a decrease in apoptosis in the border area.

We subsequently examined in a preclinical study whether or not G-CSF treatment is effective in preventing cardiac remodeling after MI in large animals [9]. An MI was produced by ligation of the left anterior descending coronary artery in swine. G-CSF (10 μg/kg/day) was injected subcutaneously, starting 24 hours after the ligation for 7 days. Subsequent echocardiographic examination at 4 weeks after the MI revealed that G-CSF treatment improved cardiac function and reduced LV remodeling [9]. In the ischemic region, the number of apoptotic endothelial cells was less and the number of vessels increased in the G-CSF treatment group compared to the control group. In addition, vascular endothelial growth factor was expressed more abundantly and Akt was activated to a greater extent in the ischemic region of the G-CSF treatment group than in the control group. Akt has been reported to play an important role in cell survival and angiogenesis, and seems to play an important role in the cardioprotective effects of G-CSF. These findings suggest that G-CSF reduces the extent of cardiac dysfunction and remodeling after MI in large animals. It is noteworthy that as early as 1 week after MI, there was a difference in LV function between the G-CSF treatment group and the control group. The finding was not explained simply by G-CSF-induced mobilization of BMSCs into the injured myocardium. So we speculated that G-CSF could beacting directly on the myocardium in the infarcted heart. As mentioned above, it has been reported that the G-CSF receptor (G-CSFR) is expressed on various blood cells, but whether G-CSFR is expressed on cardiomyocytes has yet to be determined. We detected the expression of G-CSFR mRNA in both the adult mouse heart and the cultured neonatal mouse cardiomyocyte by the reverse transcription–polymerase chain reaction [10]. Furthermore, we recognized the expression of G-CSFR protein in neonatal rat cardiomyocytes by immunocytochemical analysis. We next examined whether G-CSF could induce the activation of intracellular signaling molecules in cardiomyocytes. G-CSF (100 ng/mL) significantly activated Jak2, STAT1, and STAT3 in a dose-dependent manner [10]. These results suggest that G-CSFR is expressed on cardiomyocyte and that G-CSF induces the activation of signaling molecules through G-CSFR.

Other groups also examined the effects of G-CSF on the myocardium of MI hearts in animal models. Minatoguchi et al. demonstrated that G-CSF prevented cardiac remodeling and ventricular dysfunction at 3 months in an ischemia-reperfusion rabbit model [7]. G-CSF increased the number of macrophages in the infarcted area at 2 days after MI and the expression levels of MMP-1 and MMP-9 in the ischemic region at 7 days after MI. They concluded that G-CSF had beneficial effects on MI hearts through acceleration of the healing process and by myocardial regeneration. Sugano et al. reported that G-CSF attenuated early ventricular expansion after AMI in rats [11]. The expression levels of TGF-β and procollagen type I and type III mRNA in the infarcted area at 3 days were higher in the G-CSF group than in the control group. Accumulation of collagen in the infarcted area at 7 days was more prominent in the G-CSF group than in the control group. Kawada et al. demonstrated in mice with MIs that G-CSF mobilized mesenchymal stem cells from the bone marrow into the infarcted area, where they subsequently differentiated into cardiomyocytes [8]. Sesti et al. investigated whether G-CSF and SCF could promote myocardial regeneration and improve LV function after coronary artery occlusion in rats [16]. An improvement in LV function at 8 weeks after infarction compared to controls was observed. However, the investigators found no evidence of new muscle cells in the infarct area. They concluded that G-CSF may have a direct stimulatory effect on the LV without influencing scar formation. In contrast to these reports, other experimental studies did not recognize the beneficial effects of G-CSF in an AMI model [17, 18]. The reason for this discrepancy is not yet understood.

Recently, the results of the REVIVAL-2 study were reported [19], where the effects of G-CSF were compared to placebo in 114 patients diagnosed with ST-segment elevation MI, who had successful reperfusion by PCI within 12 hours after onset of symptoms. In the study, patients were randomly assigned to receive placebo or 10 μg/kg of G-CSF subcutaneously for 5 days. Six months after the intervention, the investigators found that G-CSF treatment had no influence on infarct size or LV function. However, the treatment with G-CSF was started from 5 days after AMI. We reported that the benefecial effects of G-CSF were reduced when the treatment was delayed to day 3 after AMI in micc model [10]. The timing of the treatment with G-CSF should be critical to obtain the beneficial effects. In the future, these results need to be confirmed in larger studies and the effects of G-CSF in larger and smaller infarcts need to be examined, since only patients with extensive injury may respond.

STAT3 as a key molecule in G-CSF-induced cardioprotective effects

As apoptotic cell death is increased immediately after AMI, we examined the effect of G-CSF on hydrogen peroxide (H_2O_2)-induced apoptosis of cardiomyocytes. Rat neonatal cardiomyocytes were exposed to H_2O_2 and apoptotic cell death was detected by annexin V staining. Pretreatment with G-CSF significantly decreased the rate of H_2O_2-induced apoptosis of cardiomyocytes [10]. Although expression levels of antiapoptotic proteins such as Bcl-2 and Bcl-xL, which are target molecules of the Jak-STAT pathway, were decreased after stimulation with H_2O_2, this reduction was inhibited by pretreatment with G-CSF. AG490, an inhibitor of Jak2, abolished the up-regulation of Bcl-2 expression level by G-CSF. Adenovirus transfection of dominant-negative STAT3 (dnSTAT3) to cardiomyocytes inhibited the protective effect of G-CSF [10]. These results suggest that G-CSF may inhibit apoptosis of cardiomyocytes through the Jak2-STAT3 pathway.

STAT3 is a member of the STAT family of transcription factors and is activated by numerous growth factors and cytokines including IL-6, leukemia inhibitory factor (LIF), and cardiotrophin-1 through the shared receptor glycoprotein 130. Upon receptor activation, STAT3 is phosphorylated and activated by Jak1 or Jak2. Several studies demonstrated that STAT3 protects the heart against pathophysiologic stress such as ischemia, mechanical stress, and cytotoxic agents. Therefore, we next studied transgenic mice with cardiac-specific overexpression of dnSTAT3 (dnSTAT3-TG) to see whether STAT3 is involved in the cardioprotective effects of G-CSF *in vivo*. Although there were no significant differences in LV function and size between wild-type (WT) mice and dnSTAT3-TG mice at baseline, the cardioprotective effects of G-CSF on post-MI hearts were abolished in dnSTAT3-TG mice [10]. Expression levels of Bcl-2 and Bcl-xL in MI hearts of the WT, but not of the dnSTAT3-TG mice were increased by G-CSF treatment. Although G-CSF similarly increased the number of stem cells (double-positive cells for c-kit and Sca-1) in peripheral blood in WT and dnSTAT3-TG mice, G-CSF did not increase cardiac homing of bone marrow cells 2 weeks after MI in both groups. G-CSF did not affect the number of cardiac stem cells, which exist in the Sca-1-positive populations of adult myocardium, in post-MI hearts of both the WT and the dnSTAT3-TG mice. These results suggest that the beneficial effects of G-CSF on post-MI hearts may be attributed to a direct action on the myocardium rather than mobilization and differentiation of stem cells.

Various studies have shown the involvement of STAT3 in cardiovascular diseases. It has been demonstrated that the expression level of STAT3 in the myocardium is significantly decreased in patients with end-stage dilated cardiomyopathy [20]. Transgenic mice with cardiac-specific overexpression of constitutively active STAT3 (caSTAT3-TG) presented an increase in capillary density accompanied by an enhanced expression of vascular endothelial growth factor in the heart [21]. Infarct size in the hearts of caSTAT3-TG mice was significantly reduced compared with WT mice after ischemia-reperfusion injury [22]. Reactive oxygen species (ROS) generated in the reperfused myocardium led to cell damage and heart failure. The level of ROS production was less in the hearts of caSTAT3-TG mice than WT mice, and ROS scavengers, metallothionein1 and metallothionenin2, were up-regulated in the hearts of caSTAT3-TG mice [22]. These results raise the possibility that G-CSF may increase coronary blood flow and

ameliorate heart failure through up-regulation of STAT3, even in a nonischemic cardiomyopathy such as idiopathic dilated cardiomyopathy.

It is well known that long-term therapy with doxorubicin, an antitumor drug, induces irreversible cardiomyopathy [23]. Doxorubicin-induced cardiomyopathy is histopathologically associated with a loss of myofibrils, distension of the sarcoplasmic reticulum, and vacuolization of cytoplasm. Although redox, ROS, and mitochondrial dysfunction are proposed as the pathogenesis of doxorubicin-induced cardiac toxicity, the precise mechanism has not been fully elucidated. It was previously reported that treatment with doxorubicin reduced the STAT3 mRNA level of myocardium in mice and led to an increase in mortality due to CHF [24]. Overexpression of STAT3 in cardiomyocytes caused not only cardiac hypertrophy but also protection from doxorubicin-induced cardiac toxicity [24]. These results suggest that G-CSF may protect the heart from doxorubicin cardiotoxicity through up-regulation of STAT3 in cardiomyocytes. We have recently reported that intramuscular injection of LIF plasmid DNA induces regeneration of the myocardium and prevents cardiac dysfunction after AMI in mice [25]. As LIF is also known to activate STAT3 through glycoprotein 130, our results reinforce the hypothesis that STAT3 may be important in providing cardioprotection from pathophysiologic stress.

Postconditioning-like effects of G-CSF

We have recently examined whether G-CSF administered at the onset of reperfusion has acute postconditioning-like effects on the extent of myocardial ischemia-reperfusion injury [26]. G-CSF phosphorylated and activated ERK, Jak2, STAT3, Akt, and eNOS and significantly reduced the infarct size. Since a Jak2 inhibitor AG490 inhibited G-CSF-induced phosphorylation of Jak2, STAT3, Akt, and eNOS, but not ERK, and a PI3K inhibitor LY294002 suppressed G-CSF-induced phosphorylation of Akt and eNOS, but not Jak2, STAT3, and ERK, the signals are activated by the order Jak2 > PI3K > Akt > eNOS, and the signaling pathways of ERK may be different. G-CSF increased nitric oxide production in reperfused hearts and its effect was inhibited by L-NAME. Furthermore, the reduction of infarct size afforded by G-CSF administration was completely abolished in the presence of AG490, LY294002, and L-NAME, but not by PD98059 [26]. These results suggest that G-CSF acts directly on the myocardium during ischemia-reperfusion injury and has cardioprotective effects even if G-CSF administration is started after reperfusion and that G-CSF-induced activation of Akt-eNOS and production of nitric oxide are important for its acute cardioprotective effects.

Transcriptional regulation by the phosphorylation of Jak2-STAT3 pathway is one of the key mechanisms in G-CSF-mediated cardioprotection against MI in the chronic stage, whereas the Akt-eNOS pathway may be very important in the acute stage. eNOS has been reported to be phosphorylated by Akt, and activated eNOS-producing nitric oxide has been reported to play a pivotal role in the cardioprotection of preconditioning by preserving ischemic blood flow and attenuating platelet aggregation and the neutrophil–endothelium interaction following ischemia-reperfusion [27]. Nitric oxide is known to be the trigger for ischemic preconditioning, especially during the late phase, and activates downstream pathways including protein kinase G, mitochondrial KATP channels, free radicals, and protein kinase C. However, it remains unknown whether nitric oxide acts as the trigger for postconditioning as well as preconditioning. It has been recently reported that postconditioning inhibits the opening of the mitochondrial permeability transition pore (mPTP), which is involved in ischemia-reperfusion injury [28], and that nitric oxide inhibits mPTP opening [27]. Since glycogen synthase kinase-3β (GSK3β), another downstream molecule of Akt, has been reported to induce opening mPTP [29], GSK 3β may also be involved in the G-CSF-induced inhibition of ischemia-reperfusion injury. Additional studies are needed to clarify the downstream actions of nitric oxide in the postconditioning-like effects of G-CSF against ischemia-reperfusion injury. The present study suggests that G-CSF can be used as a novel and valuable postconditioning agent.

Effects of G-CSF on ischemic cardiomyopathy

Ischemic cardiomyopathy is the leading cause of CHF in many countries [30]. In the hibernating myocardium, contractile function of the LV

is depressed because of reduced myocardial perfusion [31]. The viable but dysfunctional myocardium could be reversed by restoration of myocardial blood flow [32]. Currently, pharmacological therapy that reduces myocardial oxygen demand is used to treat chronic myocardial ischemia, if interventional therapies such as coronary artery bypass grafting and PCI are not feasible. Although interventional therapies these are performed to increase blood supply to the ischemic region, many patients with ischemic cardiomyopathy cannot be treated with these procedures because of severe and diffuse coronary atherosclerosis. Therefore, novel strategies to treat such patients are eagerly sought after. We examined whether G-CSF treatment has effects on the chronic hibernating myocardium in swine [33]. G-CSF was found to improve the cardiac function of chronic myocardial ischemia through a decrease in fibrosis and apoptotic death and by an increase in vascular density in the ischemic region [33]. In ischemic myocardium, the activity of Akt decreased, but this activity was increased by the treatment with G-CSF. We have not examined whether G-CSF-induced mobilization of these cells plays an important role in the beneficial effects on chronic myocardial ischemia, but there is a possibility that both direct effects on the myocardium and indirect effects such as mobilization and homing of stem cells may be involved.

G-CSF could prevent the progression of LV remodeling in hibernating myocardium as well as in the infarcted myocardium. There are many patients with global ischemic cardiomyopathy in whom the culprit lesions of coronary arteries are not eligible for PCI in the clinical setting. G-CSF may become a promising therapy for those patients.

Conclusion and future prospects

Many experimental data suggest that treatment with G-CSF might become a novel therapeutic strategy for AMI. It is conceivable that the antiapoptotic and angiogenic effects of G-CSF are effective for reducing adverse LV remodeling following an AMI (Figure 14.2). However, Kang et al. reported that G-CSF treatment (10 μg/kg for 4 days before PCI) increased the rate of in-stent restenosis at the culprit lesion in patients with AMI or old MI who underwent elective PCI [34]. Although the size of their clinical study was very small and the mechanism by which G-CSF accelerated restenosis was unclear, the result

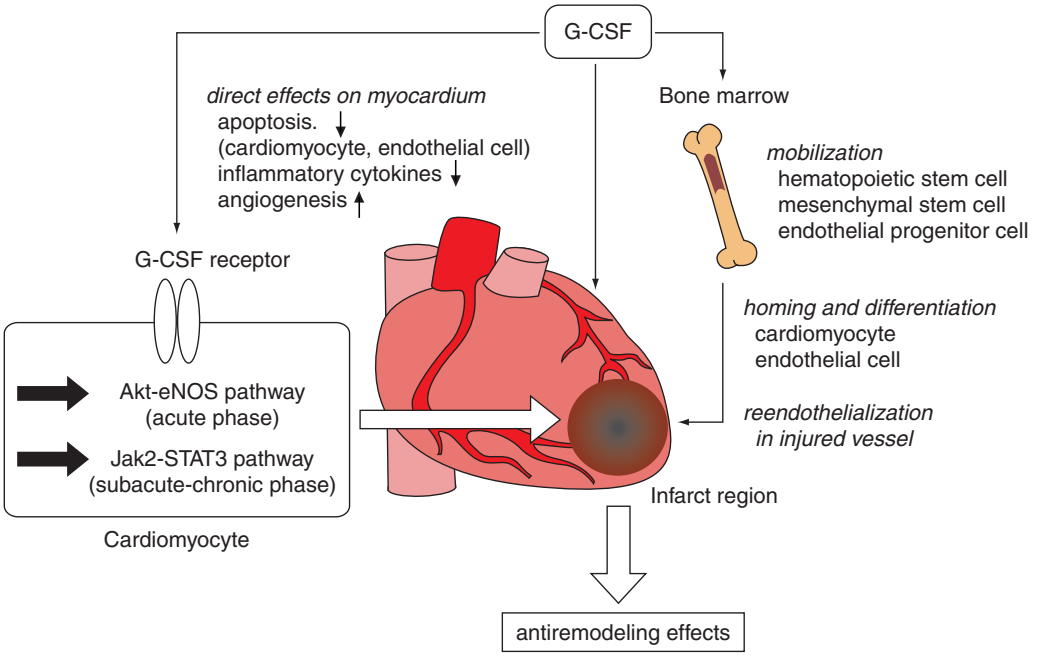


Figure 14.2 Hypothetical scheme demonstrating the mechanisms of antiremodeling effects by G-CSF.

attracted much attention regarding the safe use of G-CSF for patients with atherosclerosis. Investigators have recently examined the effects of G-CSF on neointimal formation after vascular injury in the animal model. G-CSF significantly prevented an increase in neointima/media ratio and accelerated the reendothelialization of denuded arteries in mice [35], rats [36], and rabbits [37].

When we perform clinical trials in the future to assess the feasibility and safety of novel therapies, including cell therapy, gene therapy, and cytokine therapy for coronary heart disease, we must strictly determine the inclusion criteria of patients. Patients with severe coronary lesions or unstable vital signs are not eligible for those trials. Although recent clinical trials suggest the feasibility and safety of G-CSF treatment in AMI, its long-term safety has not yet been established. Additionally, it has not been clarified what the dose of G-CSF should be and when the treatment should be started. Further studies are needed to determine the most effective and safe regimen of G-CSF treatment.

References

1 Jessup M, Brozena S. Heart failure. N Engl J Med 2003; 348:2007–2018.

2 Nian M, Lee P, Khaper N. et al. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004; 94:1543–1553.

3 Papayannopoulou T. Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood 2004; 103:1580–1585.

4 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410:701–705.

5 Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001; 98:10344–10349.

6 Ohtsuka M, Takano H, Zou Y, et al. Cytokine therapy prevents left ventricular remodeling and dysfunction after myocardial infarction through neovascularization. FASEB J 2004; 18:851–853.

7 Minatoguchi S, Takemura G, Chen XH, et al. Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation 2004; 109:2572–2580.

8 Kawada H, Fujita J, Kinjo K, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 2004; 104:3581–3587.

9 Iwanaga K, Takano H, Ohtsuka M, et al. Effects of G-CSF on cardiac remodeling after acute myocardial infarction in swine. Biochem Biophys Res Commun 2004; 325:1353–1359.

10 Harada M, Qin Y, Takano H, et al. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat Med 2005; 11:305–311.

11 Sugano Y, Anzai T, Yoshikawa T, et al. Granulocyte colony-stimulating factor attenuates early ventricular expansion after experimental myocardial infarction. Cardiovasc Res 2005; 65:446–456.

12 Takano H, Hasegawa H, Nagai T, et al. Implication of cardiac remodeling in heart failure: mechanisms and therapeutic strategies. Intern Med 2003; 42:465–469.

13 Jugdutt BI. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? Circulation 2003; 108:1395–1403.

14 Abbate A, Biondi-Zoccai GG, Baldi A. Pathophysiologic role of myocardial apoptosis in post-infarction left ventricular remodeling. J Cell Physiol 2002; 193:145–153.

15 Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001; 344:1750–1757.

16 Sesti C, Hale SL, Lutzko C, Kloner RA. Granulocyte colony-stimulating factor and stem cell factor improve contractile reserve of the infarcted left ventricle independent of restoring muscle mass. J Am Coll Cardiol 2005; 46:1662–1669.

17 Norol F, Merlet P, Isnard R, et al. Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model. Blood 2003; 102:4361–4368.

18 Deten A, Volz HC, Clamors S, et al. Hematopoietic stem cells do not repair the infarcted mouse heart. Cardiovasc Res 2005; 65:52–63.

19 Zohlnhofer D, Ott I, Mehilli J, et al. REVIVAL-2 Investigators. Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. JAMA 2006; 295:1003–1010.

20 Podewski EK, Hilfiker-Kleiner D, Hilfiker A, et al. Alterations in Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling in patients with end-stage dilated cardiomyopathy. Circulation 2003; 107:798–802.

21 Osugi T, Oshima Y, Fujio Y, et al. Cardiac-specific activation of signal transducer and activator of transcription 3 promotes vascular formation in the heart. J Biol Chem 2002; 277:6676–6681.

22 Oshima Y, Fujio Y, Nakanishi T, et al. STAT3 mediates cardioprotection against ischemia/reperfusion injury through metallothionein induction in the heart. Cardiovasc Res 2005; 65:428–435.
23 Wallace KB. Doxorubicin-induced cardiac mitochondrionopathy. Pharmacol Toxicol 2003; 93:105–115.
24 Kunisada K, Negoro S, Tone E, et al. Signal transducer and activator of transcription 3 in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy. Proc Natl Acad Sci USA 2000; 97:315–319.
25 Zou Y, Takano H, Mizukami M, et al. Leukemia inhibitory factor enhances survival of cardiomyocytes and induces regeneration of myocardium after myocardial infarction. Circulation 2003; 108:748–753.
26 Ueda K, Takano H, Hasegawa H, et al. G-CSF directly inhibits myocardial ischemia-reperfusion injury through Akt-eNOS pathway. Arterioscler Thromb Vasc Biol 2006; 26: e108–e113.
27 Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. Cardiovasc Res 2004; 61:402–413.
28 Argaud L, Gateau-Roesch O, Raisky O, et al. Postconditioning inhibits mitochondrial permeability transition. Circulation 2005; 111:194–197.
29 Juhaszova M, Zorov DB, Kim SH, et al. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004; 113:1535–1549.
30 Camici PG. Hibernation and heart failure. Heart 2004; 90:141–143.
31 Heusch G, Schulz R, Rahimtoola SH. Myocardial hibernation: a delicate balance. Am J Physiol Heart Circ Physiol 2005; 288:H984–H999.
32 Wijns W, Vatner SF, Camici PG. Hibernating myocardium. N Engl J Med 1998; 339:173–181.
33 Hasegawa H, Takano H, Iwanaga K, et al. Cardioprotective effects of granulocyte colony-stimulating factor in swine with chronic myocardial ischemia. J Am Coll Cardiol 2006; 47:842–849.
34 Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004; 363:751–756.
35 Yoshioka T, Takahashi M, Shiba Y, et al. Granulocyte colony-stimulating factor (G-CSF) accelerates reendothelialization and reduces neointimal formation after vascular injury in mice. Cardiovasc Res 2006; 70: 61–69.
36 Takamiya M, Okigaki M, Jin D, et al. Granulocyte colony-stimulating factor-mobilized circulating c-Kit+/Flk-1+ progenitor cells regenerate endothelium and inhibit neointimal hyperplasia after vascular injury. Arterioscler Thromb Vasc Biol 2006; 26:751–757.
37 Hasegawa H, Takano H, Ohtsuka M, et al. G-CSF prevents the progression of atherosclerosis and neointimal formation in rabbits. Biochem Biophys Res Commun 2006; 344: 370–376.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

15 CHAPTER 15

IGF-1, muscle progenitors, and heart failure

Nadia Rosenthal, PhD, *Nadine Winn,* PhD, *& Maria Paola Santini,* PhD

Introduction

During the last decade, considerable excitement has been generated by the experimental observations that endogenous or supplementary progenitor cells have the potential to restore the form and function of damaged tissues. Nowhere is application of this potential more welcome than in the treatment of cardiovascular disease, where the use of stem cells is currently being explored. The human heart must maintain its structure and function for decades, yet its restricted regenerative capacity is inadequate to respond to injury, when a rapid response is required to repair damage to the muscle wall and to maintain adequate blood flow to the rest of the body. In addition to the muscle loss that accompanies myocardial infarction and ischemia-reperfusion injury, interruption of the coronary blood supply results in further cell death and fibrotic scar formation at the cost of functional muscle. The remaining cardiomyocytes must then respond to an increased cardiac wall stress by undergoing pathological hypertrophy, leading to decompensated function and congestive heart failure, an overwhelming cause of morbidity and mortality in the industrialized world.

Although mammalian organs and tissues differ in their regenerative capacity, a common set of problems remains to be solved for regeneration biologists. What can we learn from the mechanisms of cardiac regeneration in the embryo or in organisms where it occurs naturally? The heart is the first fully differentiated structure to form and function in the embryo, arising from specialized mesodermal precursors and growing through the rapid cell division of already contracting cardiac myocytes. However, this phase of abundant proliferation ends soon after birth, when the capacity for heart muscle to reenter the cell cycle appears to be largely lost, and increases in myocardial mass are achieved almost exclusively through cellular hypertrophy.

The regenerative inadequacy of the mammalian heart stands in marked contrast to the robust proliferative capacity and rapid cardiac regeneration in other vertebrates such as the newt and zebrafish [1, 2], underscoring the dramatic evolutionary variability of cardiac regeneration [3]. The mammalian heart is capable of homeostasis, maintaining cell renewal throughout life [4, 5], but the relative rarity of adult cardiac progenitor cells may impose severe limits on repair. In regeneration-competent tissues, individual cells constantly undergo structural renewal by replacing their molecular components and are themselves periodically replaced with new cells; but it is not clear to what extent this self-renewal relies on the morphogenetic patterning that forms the tissue in the embryo. Nevertheless, the hope is that nonregenerating human tissues maintain a latent capacity for regeneration, once the impediments that limit this capacity in the adult are identified and circumvented.

Cell-based cardiac therapy

The traditional paradigm of the mammalian heart as an organ incapable of regeneration has been put into question by the recent finding of multipotent

cardiac stem cells in the adult heart, capable of regenerating myocytes and coronary vessels throughout life [6]. These studies provide a plausible explanation for the inherent self-renewing capacity of the adult organ in an unchallenged state, but major questions regarding cardiac physiology remain to be resolved. If cardiac muscle possesses a stem cell compartment, it is not clear why the heart fails to regenerate under pathological conditions. Either the resident cardiac stem cells are too rare or intrinsically incapable of repairing major damage, or perhaps the damaged myocardium presents a prohibitive environment for their activation. It is possible that the mere presence of endogenous stem cells may not be sufficient to guarantee cardiac regeneration and that the presence of appropriate stimuli and factors are necessary to provide a permissive environment that permits stem-cell-mediated cardiac repair.

Cell-based strategies for restoration of function in heart failure have been developed to overcome this inherent barrier to effective regeneration. However, the clinical application of these strategies has been difficult. Aside from the ethical issues surrounding the use of xenographic embryonic stem cells, they can induce an immune response, which may pose a serious impediment to their long-term survival in the host myocardium. A more practical scenario relies on autologous adult progenitor cells, including bone-marrow-derived, circulating, or organ-specific progenitor cells. Cells from fat, skeletal muscle, or the heart itself [7–10] have been shown to improve myocardial function in experimental heart failure models, prompting several clinical trials targeting patients with chronic heart failure, using skeletal myoblasts or bone-marrow-derived cells [10]. Injection or implantation of autologous or heterologous regeneration-competent progenitor cells has been moderately successful at improving left ventricular functions [11, 12], although the underlying mechanisms for this benefit remain obscure and the incidence of ventricular arrhythmias after direct injection of cells into the myocardium has caused considerable alarm [13, 14]. In addition, the relatively poor survival rate of introduced cells suggests that the appropriate tissue environment is critical for the productive reconstitution of injured tissue. It is likely that fibrotic tissue formation leading to cardiac functional impairment precludes the effective repopulation of the injured area by exogenous cells. Thus, improving the tissue environment may broaden the scope of adult cell types that can participate in functional restoration of the myocardium.

IGF-1 isoforms—a complex growth factor family

An emerging strategy to provide a better regenerative environment in the damaged heart involves the use of growth factors. In particular, insulin-like growth factor-1 (IGF-1) has long been implicated in the control of cardiac growth *in vitro* and *in vivo* [15]. Much research effort has been invested to evaluate the variety of effects that IGF-1 has on different cells types and tissues of the body at all stages of pre- and postnatal development. An impressive body of knowledge has been accumulated, but surprisingly the existence of multiple isoforms generated from the IGF-1 gene was not taken into account in the majority of the studies on IGF-1 function, despite their discovery over 20 years ago.

Unlike the closely related insulin gene, the mammalian IGF-1 gene locus encodes multiple precursor proteins, which vary in the N-terminal signal peptide sequence and the C-terminal E-domain amino acid sequences (Figure 15.1). The unprocessed precursor peptides undergo post-translational protease cleavage to generate mature IGF-1—a 70 amino acid single chain polypeptide with three intrachain disulfide bridges. Mature IGF-1 differs from insulin by retention of the C-domain, by a short extension of the A-domain to include a novel domain D, and by the presence of variable C-terminal extension peptides (E peptides). IGF-1 shares 62% homology with proinsulin and 48% homology with insulin [16]. Among mammalian species the primary structure of IGF-1 is highly conserved: canine [17], bovine [18], ovine [19], porcine [20], and human [16] IGF-1s are identical, whereas rat [21] and mouse [22] IGF-1s differ from that seen in humans by three and four amino acids, respectively.

Two additional protein products are produced by posttranslational cleavage of IGF-1 precursor protein, identified in the human brain [23]. A truncated IGF-1 isoform (−3N:IGF-1) lacks the first three amino acids from the NH_2-terminal end of

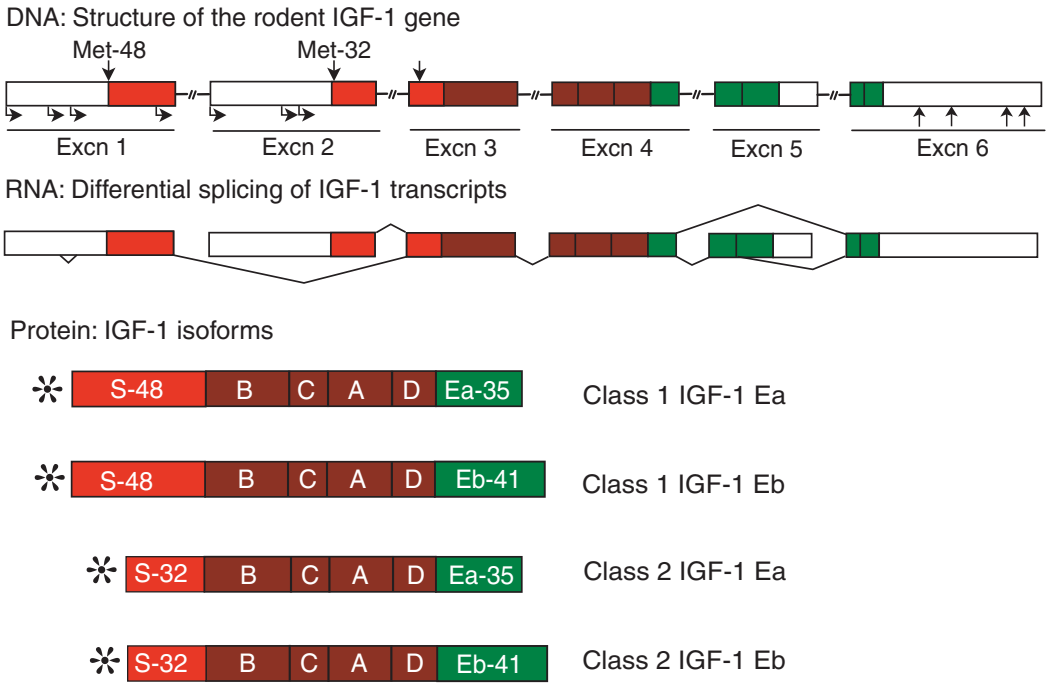


Figure 15.1 Genomic organization and expression of the rodent IGF-1 locus. The mammalian single-copy IGF-1 gene contains six exons, separated by five introns. Exons 1 and 2 contain multiple transcription start sites and encode distinct, mutually exclusive 5′-untranslated regions as well as N-terminal signal peptide sequence. Exons 3 and 4 encode the IGF-1 B, C, A, and D and part of the E-domain. Exon 5 and exon 6 each encode distinct extension peptides (E peptides), termination codons, and 3′-untranslated regions. Transcription of the IGF-1 gene is complex, through use of alternative promoters and transcription start sites located in leader exons, alternative posttranscriptional exon splicing, and use of different polyadenylation sites. These multiple IGF-1 mRNA transcripts encode different IGF-1 precursors, which undergo further posttranslational cleavage to release the biologically active mature IGF-1.

the B-domain, has low binding affinity to IGF-binding proteins, and displays enhanced biological (neurotrophic) effects, which are mediated through the IGF-1 receptor [24, 25]. Another product from this posttranslational modification of IGF-1 precursor protein is the tripeptide glycyl-prolyl-glutamate (gly-pro-glu), which corresponds to the amino terminal end of mature IGF-1 [23]. In the central nervous system, gly-pro-glu modulates neurotransmitter release via the N-methyl-D-aspartate type of glutamate receptor, although the relevance of these posttranslational modifications to the function of IGF-1 in the heart is currently unknown.

It has been suggested that alternate splicing and differential promoter usage leads to the generation of different signal peptides that could affect the precise N-terminal cleavage site of the signal peptide [26] at a position three amino acids downstream of the usual cleavage site to produce the N-terminal truncation of the B-domain of mature or pro-IGF-1 [27, 28]. The cleavage of three N-terminal amino acids (gly-pro-glu) results in a truncated des [1–3] IGF-1 peptide [25]. Different signal peptides have also been implicated in the determination of extension peptide a glycosylation *in vitro* [29].

Tissue distribution of IGF-1 transcripts varies in both the signal peptide and E peptide coding regions. In rodents, exon 1 transcripts predominate in the liver, the primary source of circulating IGF-1 [30]. During postnatal development and in adult, expression of exon 2 transcripts is more growth hormone (GH)-dependent than expression of exon 1 transcripts [31, 32]. Exon 2 transcripts appear later in postnatal development, increasing especially at the onset of GH-dependent linear growth, and their appearance coincides with that of circulating IGF-1

Table 15.1 Summary of contrasting muscle phenotypes of IGF-1 transgenic mice*.

	Circulating IGF-1	*Muscle hypertrophy*	*Muscle regeneration*
Rskα-actin/hIGF-1 [37]	Increased	No	No change
MLC/mIGF-1 [38]	No change	Yes	Enhanced

*Also see Ref. [39].

[30]. Notably, in the heart, IGF-1 transcription derives only from the third out of four possible exon 1 start sites, whereas exon 2-containing transcripts are undetectable [33]. IGF-1 mRNAs encoding the Ea-peptide are the major transcripts found in extrahepatic tissues, including the heart. Thus, it appears that transcripts encoding the Class 1 signal peptide and the Ea-peptide expressed in extrahepatic tissues such as the heart are less GH-responsive, encoding an autocrine/paracrine form of IGF-1.

Only recently has the field begun to appreciate that the different IGF-1 isoforms have specialized functions, which might explain the pleiotropic effects of IGF-1. Indeed, injury of adult mammalian tissues induces transient production of these IGF-1 isoforms, which purportedly control cell growth, survival, and differentiation [34]. By contrast, circulating IGF-1 produced largely by the adult liver has been implicated in the restriction of lifespan [35, 36].

With an increasing research focus on the potential therapeutic application of IGF-1 in treating skeletal and cardiac muscle diseases, the need for evaluating IGF-1 isoform function has become a crucial prerequisite for developing IGF-1-based therapies. Specifically, very little is known about the intra- and extracellular processing of IGF-1 precursor peptides. It is not clear whether the variable signal peptides encoded in the first exons of the IGF-1 gene locus differentially locate the gene product or deliver it via a different cellular compartment. Similarly, the function and fate of the C-terminal E peptides has not been extensively studied.

Growth factor therapy for muscle regeneration

The importance of IGF-1 isoforms and their precursor components in the biological effects of IGF-1 is illustrated by contrasting reports in which overexpression of different IGF-I transgenes was directed to the skeletal muscle (Table 15.1). The major role in the growth, remodeling, and regeneration in skeletal muscle is played by satellite cells—a quiescent population of myogenic cells that reside between the basal lamina and plasmalemma and are rapidly activated in response to appropriate stimuli. In mice carrying a muscle-specific IGF-1 transgene lacking a native signal peptide or E peptide, truncated IGF-1 protein was released into the circulation, but had no beneficial effect when tested on muscle regeneration in autotransplanted whole muscle grafts, myofibre atrophy following sciatic nerve transaction, or sarcolemmal damage and myofibre necrosis in dystrophic muscle [37].

In contrast, the dramatic regenerative properties of a naturally occurring isoform, mIGF-1, in skeletal muscle and its dramatic promotion of cell survival and renewal in senescent muscle have been amply documented [38, 40, 41]. Expressed as a muscle-specific transgene or on a viral vector, mIGF-1 elicits a striking increase in skeletal muscle mass and strength, a rapid restoration of injured muscle, reducing scar formation. In response to injury, local production of mIGF-1 in transgenic mouse muscles results in an enhanced activation of satellite cells, as shown by a rapid induction of Pax7, a satellite cell marker [41]. To determine whether bone-marrow-derived cells could also contribute to mIGF-1-mediated regeneration, mIGF-1 mice were lethally irradiated to ablate native bone marrow and then were reconstituted with donor bone marrow carrying a GFP (green fluorescent protein) transgene driven by the c-kit promoter. By tracking transplanted bone-marrow-derived cells to skeletal muscle early in the regeneration process, we observed a fourfold increase in GFP-positive cells migrating to the injured muscle of mIGF-1 transgenic mice as compared to wild-type littermates [38]. These results indicate that local mIGF-1 induces the production of signals to increase the mobilization of uncommitted cell subsets in the bone marrow, which

migrate to sites of tissue damage and participate either directly or indirectly in the regeneration process.

The dramatic increase in progenitor cells in mIGF-1 transgenic injured muscles was ablated by the administration of 5-FU—a potent repressor of the cell cycle—which did not affect the abundance of nonproliferative cells at the site of injury. This is consistent with the effect of mIGF-1 transgene expression in primary muscle cultures, which contained an expanded myogenic population in response to muscle damage, with 50% more myoblasts seen than in wild-type control cultures. Thus, regenerative cell populations increased by the presence of mIGF-1 rely on proliferative capacity [38]. Finally, skeletal myoblasts isolated from mIGF-1 transgenic muscle stimulated the conversion of bone marrow cells to a myogenic lineage when cocultured with bone marrow stem cell subpopulations [38]. These studies emphasize the importance of IGF-1 as an inductive signal, which increases the efficiency of stem cell action and improves tissue regeneration.

Another IGF-1 isoform, Class 1 IGF-1Eb, has also been shown to enhance the integration of bone-marrow-derived cells into skeletal muscle *in vivo* and to promote fusion of a subset of bone-marrow-derived cells, myelomonocytic precursors, to cocultured myotubes *in vitro* [42]. These results further highlight the crucial role of IGF-1 isoforms in tissue repair.

In summary, the marked contrast between phenotypes elicited by overexpression of different IGF-1 isoforms underscores the importance of further research on the molecular underpinnings of their action. In contrast to artificially truncated forms, the mIGF-1 isoform is a powerful enhancer of local regeneration, mediating the recruitment of bone marrow cells to sites of tissue damage and their contribution to muscle repair. Enhanced mobilization and homing of local as well as bone-marrow-derived progenitor cells by local mIGF-1 is triggered by damage, and presumably contributes to increases in muscle mass, strength, and resistance in age-related atrophy and degenerative disease. However, it is likely that other naturally accruing IGF-1 isoforms may play equally important roles in tissue homeostasis and regeneration (N. Winn and N. Rosenthal, manuscript in preparation), particularly in the context of the myocardium. A comprehensive comparison of IGF-1 isoform function in the heart is long overdue.

Enhancing cardiac muscle regeneration

A number of recent studies have tested IGF-1 in the setting of myocardial infarction, demonstrating that this growth factor can also contribute to an improved tissue environment in the damaged heart. Cardiac stem cells and early committed cells express IGF-1 receptors and synthesize and secrete IGF-1 [43]. When fully processed IGF-1 was injected together with hepatocyte growth factor (HGF) into infarcted mouse hearts, this combination of growth factors induced the formation of new myocardium that contained arterioles, capillaries, and functionally competent myocytes that gradually increased in size, improving ventricular performance. This improvement in myocardial regeneration was achieved through stimulation of an endogenous reserve of progenitor cells that reconstituted damaged myocardium and restored cardiac function. This study demonstrates how the heart can be transformed into a regeneration-competent organ through the combined actions of growth factors that improve the local environment and activate endogenous stem cell reserves [43].

The biology that underlies these exciting observations is still not well understood. As in skeletal muscle, different IGF-1 isoforms have contrasting effects on the heart when expressed as transgenes, variously promoting cell survival or inducing prolonged hypertrophy with pathological consequences [44, 45]. To determine the extent to which supplemental expression of the mIGF-1 isoform was capable of promoting regeneration of cardiac tissue, we have generated transgenic mice in which the mIGF-1 transgene was driven by a cardiac gene promoter, to restrict expression of mIGF-1 to the mouse myocardium and exclude possible endocrine effects on other tissues. Transgenic mIGF-1 expression levels increased with age exclusively in the heart, which precociously attained adult size without perturbing function. In contrast to the characteristic progression of scar formation in wild-type hearts after myocardial infarction, mIGF-1 transgene expression induced repair of the injured tissue,

with minimal scar formation after 1 month with integrity of the posterior wall and normal echocardiographic profiles. Functional parameters of the recovered mIGF-1 hearts were also significantly improved when compared to infarcted control animals. This functional improvement was due in part to an amelioration of the cellular environment of the postinfarct heart, with marked reduction in inflammatory markers and increased periinfarct proliferative activity. These preliminary results suggest that in the mIGF-1 transgenic hearts, cardiac regeneration involves early resolution of inflammation at the site of injury to prevent scar formation, making way for the subsequent tissue replacement that restores form and function (M.P. Santini and N. Rosenthal, unpublished results). It is therefore likely that supplemental mIGF-1 improves the tissue environment through specific cell signaling pathways that reduce the fibrotic response.

The origin and identity of cells that are stimulated by IGF-1 to promote cardiac regeneration is still under debate. Our observations of increased proliferative activity, relatively late after myocardial infarction of mIGF-1 hearts, suggest that some of these cycling cells may correspond to a population of rare, small cardiomyocytes that retain the capacity to proliferate in response to damage [46–48]. Cells capable of differentiating into a myocyte in the adult heart [49, 50] could also originate through the commitment of precursor cells to the myocyte lineage. Undifferentiated precursor cells in the adult heart have been identified through cell surface proteins that mark stem cell populations in other tissues [51, 52]. In some cases, these cells give rise to clones that express biochemical markers of myocytes, smooth muscle, and endothelial cells *in vitro*, underscoring their stem-cell-like nature.

It is also possible that endothelial precursors are stimulated by mIGF-1 to contribute directly to regenerating myocardial tissue. Indeed, adult human endothelial progenitor cells, derived from peripheral blood mononuclear cells, or hematopoietic progenitor cells can convert into cardiomyocytes when cocultured with rat cardiomyocytes [53]. In these studies, cell–cell contact or an extracellular matrix-associated signaling appeared to be critical, because conditioned media from cardiomyocyte cultures was not sufficient for conversion to a cardiomyocyte phenotype.

Cycling myocardial cells induced by mIGF-1 may further derive from recently characterized cells isolated from the adult rat heart which retain stem cell characteristics [54]. These cells are self-renewing, clonogenic, and multipotent *in vitro* and *in vivo* and give rise to myocytes and smooth muscle and endothelial vascular cells. When injected into an ischemic rat heart, a population of these cells or their clonal progeny reconstitute up to 70% of the injured myocardial wall. Since this endogenous pool of primitive multipotent cells can be recruited into the regenerative process by the intramyocardial injection of a growth factor cocktail (IGF-1 and HGF), a combined cell-plus-factor strategy might be most effective for promoting translocation to the damaged area and activating growth and differentiation [43]. The blunted response to HGF or IGF-1 alone [43] has been attributed to the need for improvement of survival and promotion growth provided by IGF-1 together with the chemotactic effects of HGF. However, it is possible that fully processed IGF-1 may not maintain the full spectrum of activities seen for the endogenous forms such as mIGF-1 [38]. Dissecting the distinct functions of the various IGF-1 isoforms in promoting cardiac regeneration remains a major challenge in the field and will undoubtedly yield new approaches to growth factor enhancement of the regenerative process.

An integrated approach for the future

Although effective cell-based repair of the mammalian heart is now a feasible goal of regenerative medicine, further investigation is necessary to improve the very poor graft survival and rate of proliferation of cells after injection and to exploit their capacities to transdifferentiate, integrate, or protect the remaining myocardium. Since supplementary IGF-1 enhances myocardial regeneration following injury by enhancing endogenous repair mechanisms without affecting long-term postnatal organ function, it may constitute a clinically feasible therapeutic reagent to bypass the normal restrictions on mammalian cardiac repair. Although the evidence to date for growth factor supplementation is encouraging, it remains to be determined which combination of growth factors elicits the most effective response.

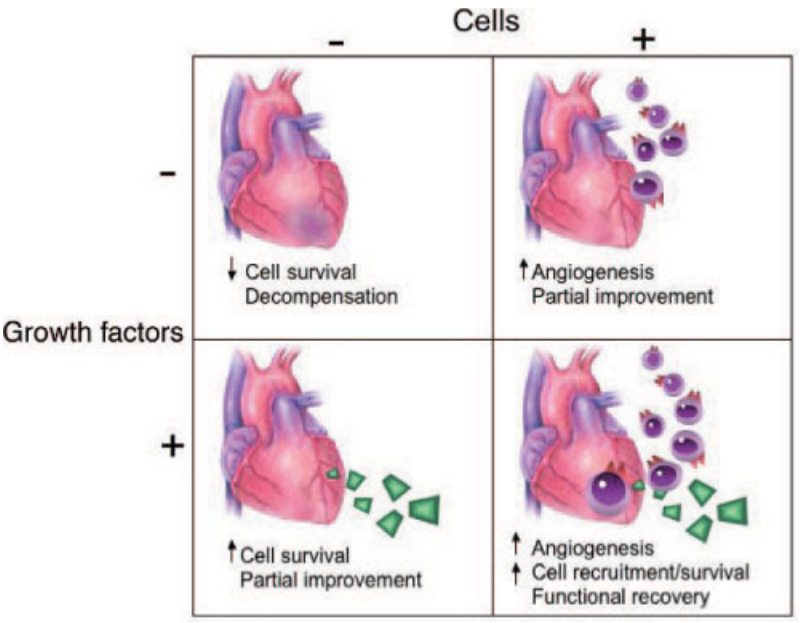


Figure 15.2 Combinatorial cell and growth factor therapies. Potential advantages of the combining cells and growth factors in cardiac repair and regeneration. In the wake of a myocardial infarction, the action of growth factors such as IGF-1 and HGF stabilizes the necrotic zone and restricts fibrosis by activation of survival signals, inducing the secretion of anti-inflammatory cytokines and chemoattractive molecules that promote contribution of progenitor cells to the areas of injury. Endogenous precursor cell pools are activated to proliferate and are recruited to the damage tissues. Increasing the progenitor cell pool through introduction of exogenous cells increases the angiogenic activity in the infarct border zone and further potentiates the repair process. The optimal identities of the cells and growth factors in each scenario remain to be determined.

Novel approaches will also be required to overcome the insufficiencies of endogenous stem cells to alleviate acute and chronic damage to mammalian cardiac tissues. The possibility that endogenous cardiac stem cells can be mobilized by growth factors to migrate from their niche within the healthy heart and help regenerate diseased myocardium has additional implications for therapeutic intervention [55]. This possibility is supported by the enhancement of recovery from nonischemic heart failure, using the current generation of implantable left ventricular assist devices [56, 57] in combination with the $\beta 2$ agonist clenbuterol, to induce a mild degree of physiologic hypertrophy, enhance electromechanical coupling, and markedly improve hemodynamic function [56, 58, 59]. The exact mechanism of regeneration in these patients is not known but could include revival of mature cells as well as migration of stem cells from the heart or bone marrow [7, 8]. Indeed, patient myocardia contained elevated levels of IGF-1 and stromal cell-derived factor-1 (SDF-1), both of which are known to enhance recruitment of stem cells [38, 60]. It is increasingly clear that an instructive tissue environment is critical and may broaden the scope of adult cell types that can participate in functional cardiac repair.

For the future, therapeutic strategies for the restoration or replacement of cardiac tissues and structures are likely to derive from combining the use of cells and growth factors (Figure 15.2). Surgical or mechanical interventions, bioengineered materials, and associated technologies may provide additional avenues for innovation. In a recent exciting proof of concept, biotinylated IGF-1, bound to self-assembling peptide nanofibers and injected into rat myocardium, provided sustained IGF-1 delivery for 28 days and increased activation of downstream Akt signaling in the myocardium. When combined with transplanted cardiomyocytes, IGF-1 delivery by biotinylated nanofibers decreased caspase-3 cleavage,

increased myocyte cross-sectional area, and improved systolic function after experimental myocardial infarction [61], demonstrating how providing the appropriate signals and environment for cells can optimize the reconstitution of the dysfunctional organ. Coordinating current advances in regenerative research will be necessary to achieve optimal modulation of the regenerative response and to devise effective strategies that address the short-, medium-, and long-term aspects of heart failure prevention and treatment.

Acknowledgments

This work was supported by grants to N. Rosenthal from the Muscular Dystrophy Association (USA), the European Union (QLRT-2001-00930), and the Fondation Leducq.

References

1 Oberpriller JO, Oberpriller JC, Matz DG, Soonpaa MH. Stimulation of proliferative events in the adult amphibian cardiac myocyte. Ann N Y Acad Sci 1995; 752:30–46.
2 Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science 2002; 298:2188–2190.
3 Brockes JP, Kumar A, Velloso CP. Regeneration as an evolutionary variable. J Anat 2001; 199:3–11.
4 Nadal-Ginard B, Kajstura J, Anversa P, Leri A. A matter of life and death: cardiac myocyte apoptosis and regeneration. J Clin Invest 2003; 111:1457–1459.
5 Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ Res 2003; 92:139–150.
6 Anversa P, Leri A, Kajstura J. Cardiac regeneration. J Am Coll Cardiol 2006; 47:1769–1776.
7 Laugwitz KL, Moretti A, Lam J, et al. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005; 433:647–653.
8 Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001; 98:10344–10349.
9 Serruys PW, Kipshidze NN. Handbook of Cardiovascular Cell Transplantation. London: Martin Dunitz, 2004.
10 Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 2005; 115:572–583.
11 Rosenthal N, Santini MP. Stem Cells and the Regenerating Heart. New York: Academic Press, 2004.
12 Rubart M, Field LJ. Cardiac regeneration: repopulating the heart. Annu Rev Physiol 2006; 68:29–49.
13 Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest 1996; 98:2512–2523.
14 Leobon B, Garcin I, Menasche P, et al. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. Proc Natl Acad Sci USA 2003; 100:7808–7811.
15 Fazio S, Palmieri EA, Biondi B, et al. The role of the GH-IGF-I axis in the regulation of myocardial growth: from experimental models to human evidence. Eur J Endocrinol 2000; 142:211–216.
16 Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 1978; 253:2769–2776.
17 Delafontaine P, Lou H, Harrison DG, Bernstein KE. Sequence of a cDNA encoding dog insulin-like growth factor I. Gene 1993; 130:305–306.
18 Francis GL, uptonFM, Ballard FJ, et al. Insulin-like growth factors-1 and 2 in bovine colostrum. Sequences and biological activities compared with those of a potent truncated form. Biochem J 1988; 251:95–103.
19 Francis GL, McNeil KA, Wallace JC, et al. Sheep insulin-like growth factors I and II: sequences, activities and assays. Endocrinology 1989; 124:1173–1183.
20 Francis GL, Owens PC, McNeil KA, et al. Purification, amino acid sequences and assay cross-reactivities of porcine insulin-like growth factor-I and -II. J Endocrinol 1989; 122:681–687.
21 Shimatsu A, Rotwein P. Mosaic evolution of the insulin-like growth factors. Organization, sequence, and expression of the rat insulin-like growth factor I gene. J Biol Chem 1987; 262:7894–7900.
22 Bell GI, Stempien MM, Fong NM, Rall LB. Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucleic Acids Res 1986; 14:7873–7882.
23 Sara VR, Carlsson-Skwirut C, Drakenberg K, et al. The biological role of truncated insulin-like growth factor-1 and the tripeptide GPE in the central nervous system. Ann N Y Acad Sci 1993; 692:183–191.
24 Carlsson-Skwirut C, Lake M, Hartmanis M, et al. A comparison of the biological activity of the recombinant intact and truncated insulin-like growth factor 1 (IGF-1). Biochim Biophys Acta 1989; 1011:192–197.
25 Sara VR, Carlsson-Skwirut C, Bergman T, et al. Identification of Gly-Pro-Glu (GPE), the aminoterminal tripeptide of insulin-like growth factor 1 which is truncated in brain, as a novel neuroactive peptide. Biochem Biophys Res Commun 1989; 165:766–771.

26 LeRoith D, Roberts CT, Jr. Insulin-like growth factor I (IGF-I): a molecular basis for endocrine versus local action? Mol Cell Endocrinol 1991; 77:C57–C61.

27 Carlsson-Skwirut C, Jornvall H, Holmgren A, et al. Isolation and characterization of variant IGF-1 as well as IGF-2 from adult human brain. FEBS Lett 1986; 201: 46–50.

28 Sara VR, Carlsson-Skwirut C, Andersson C, et al. Characterization of somatomedins from human fetal brain: identification of a variant form of insulin-like growth factor I. Proc Natl Acad Sci USA 1986; 83:4904–4907.

29 Simmons JG, Van Wyk JJ, Hoyt EC, Lund PK. Multiple transcription start sites in the rat insulin-like growth factor-I gene give rise to IGF-I mRNAs that encode different IGF-I precursors and are processed differently in vitro. Growth Factors 1993; 9:205–221.

30 Adamo ML, Ben-Hur H, Roberts CT, Jr, LeRoith D. Regulation of start site usage in the leader exons of the rat insulin-like growth factor-I gene by development, fasting, and diabetes. Mol Endocrinol 1991; 5:1677–1686.

31 Lowe WL, Jr, Roberts CT, Jr, Lasky SR, LeRoith D. Differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. Proc Natl Acad Sci USA 1987; 84:8946–8950.

32 Adamo M, Lowe WL, Jr, LeRoith D, Roberts CT, Jr. Insulin-like growth factor I messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development of the rat. Endocrinology 1989; 124:2737–2744.

33 Shemer J, Adamo ML, Roberts CT, Jr, LeRoith D. Tissue-specific transcription start site usage in the leader exons of the rat insulin-like growth factor-I gene: evidence for differential regulation in the developing kidney. Endocrinology 1992; 131:2793–2799.

34 Goldspink G. Gene expression in skeletal muscle. Biochem Soc Trans 2002; 30:285–290.

35 Anversa P. Aging and longevity: the IGF-1 enigma. Circ Res 2005; 97:411–414.

36 Longo VD, Finch CE. Evolutionary medicine: from dwarf model systems to healthy centenarians? Science 2003; 299:1342–1346.

37 Shavlakadze T, Boswell JM, Burt DW, et al. Rskalpha-actin/hIGF-1 transgenic mice with increased IGF-I in skeletal muscle and blood: impact on regeneration, denervation and muscular dystrophy. Growth Horm IGF Res 2006; 16(3):157–173.

38 Musaro A, Giacinti C, Borsellino G, et al. Stem cell-mediated muscle regeneration is enhanced by local isoform of insulin-like growth factor 1. Proc Natl Acad Sci USA 2004; 101:1206–1210.

39 Shavlakadze T, Winn N, Rosenthal N, Grounds MD. Reconciling data from transgenic mice that overexpress IGF-I specifically in skeletal muscle. Growth Horm IGF Res 2005; 15:4–18.

40 Barton-Davis ER, Shoturma DI, Musaro A, et al. Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. Proc Natl Acad Sci USA 1998; 95:15603–15607.

41 Musaro A, McCullagh K, Paul A, et al. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 2001; 27:195–200.

42 Sacco A, Doyonnas R, LaBarge MA, Hammer MM, Kraft P, Blau HM. IGF-I increases bone marrow contribution to adult skeletal muscle and enhances the fusion of myelomonocytic precursors. J Cell Biol 2005; 171:483–492.

43 Urbanek K, Rota M, Cascapera S, et al. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res 2005; 97:663–673.

44 Delaughter MC, Taffet GE, Fiorotto ML, Entman ML, Schwartz RJ. Local insulin-like growth factor-I expression induces physiologic, then pathologic, cardiac hypertrophy in trasngenic mice. FASEB J 1999; 13:1923–1929.

45 Reiss K, Cheng W, Ferber A, et al. Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice. Proc Natl Acad Sci USA 1996; 93:8630–8635.

46 Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001; 344:1750–1757.

47 Dewald O, Frangogiannis NG, Zoerlein M, Duerr GD, et al. Development of murine ischemic cardiomyopathy is associated with a transient inflammatory reaction and depends on reactive oxygen species. Proc Natl Acad Sci USA 2003; 100:2700–2705.

48 Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P. Myocyte proliferation in end-stage cardiac failure in humans. Proc Natl Acad Sci USA 1998; 95:8801–8805.

49 Muller P, Pfeiffer P, Koglin J, et al. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. Circulation 2002; 106:31–35.

50 Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. N Engl J Med 2002; 346:5–15.

51 Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA. Myogenic specification of side population cells in skeletal muscle. J Cell Biol 2002; 159:123–134.

52 Hierlihy AM, Seale P, Lobe CG, et al. The post-natal heart contains a myocardial stem cell population. FEBS Lett 2002; 530:239–243.

53 Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor

cells into functionally active cardiomyocytes. Circulation 2003; 107:1024–1032.

54 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.

55 Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005; 85:1373–1416.

56 Yacoub M. A novel strategy to maximize the efficacy of left ventricular assist devices as a bridge to recovery. Eur Heart J 2001; 22:534–540.

57 Mancini DM, Beniaminovitz A, Levin H, et al. Low incidence of myocardial recovery after left ventricular assist device implantation in patients with chronic heart failure. Circulation 1998; 98:2383–2389.

58 Terracciano CM, Hardy J, Birks EJ, et al. Clinical recovery from end-stage heart failure using left-ventricular assist device and pharmacological therapy correlates with increased sarcoplasmic reticulum calcium content but not with regression of cellular hypertrophy. Circulation 2004; 109:2263–2265.

59 Soppa GK, Smolenski RT, Latif N, et al. Effects of chronic administration of clenbuterol on function and metabolism of adult rat cardiac muscle. Am J Physiol Heart Circ Physiol 2005; 288:H1468–H1476.

60 Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003; 362:697–703.

61 Davis ME, Hsieh PC, Takahashi T, et al. Local myocardial insulin-like growth factor 1 (IGF-1) delivery with biotinylated peptide nanofibers improves cell therapy for myocardial infarction. Proc Natl Acad Sci USA 2006; 103:8155–8160.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

PART IV

Cardiac progenitor cells and heart failure

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

16 CHAPTER 16

Cardiac stem cells and diabetic cardiomyopathy

Jan Kajstura, PhD, *Nicole LeCapitaine,* PhD, *Maria Loredo,* MD, *Marco Giorgio,* PhD, *Thomas S. Mitchell,* MD, *Susanna Valentini,* MD, *Francesco Rotatori,* MD, *& Pier Giuseppe Pelicci,* PhD

Introduction

A typical feature of the diabetic heart is the development of a myopathy that deteriorates with time, independent from intercurrent vascular manifestations. This myopathy often presents itself as ventricular dilation, with relative wall thinning, and an impairment in both diastolic and systolic function [1]. The timing parameters of myocyte shortening and relengthening are prolonged and the force generation of the myocardium is reduced. These mechanical defects have their biochemical counterpart in the phosphorylation of troponin I by protein kinase C-epsilon that conditions a decrease in myofilament calcium (Ca^{2+}) sensitivity. These and other relevant findings have left unanswered the question whether these changes play a primary or secondary role in the occurrence of a diabetic myopathy. In fact, myocyte hypertrophy of various etiologies results in similar mechanical alterations and changes in regulatory proteins [2]. A major advance in our understanding of the pathophysiology of the diabetic heart was prompted by the recognition that myocyte death and reactive myocyte hypertrophy are critical determinants of cardiac pathology with diabetes and that both are mediated partly by activation of the local renin–angiotensin system (RAS). Importantly, these etiologic factors are operative in animals with insulin-dependent diabetes mellitus (IDDM; type I) and in humans with noninsulin-dependent diabetes mellitus (NIDDM; type II) [3, 4]. These data point to the cardiac RAS as the predominant cause of the onset and development of diabetic cardiomyopathy in the absence of coronary artery disease and hypertension. Hypertension potentiates the effects of diabetes on the formation of angiotensin (Ang) II-induced cell death and cellular hypertrophy [3]. Therapeutic interventions decreasing the formation of Ang II have been shown to attenuate morbidity and mortality rates in diabetic patients, and angiotensin-converting enzyme inhibition positively modifies cardiovascular events and overt nephropathy in subjects with severe diabetes [5]. Together, these observations support the notion that diabetic cardiomyopathy is an Ang II-dependent myopathy where Ang II exerts two fundamental functions: activates the death and growth of parenchymal cells [3, 4]. In the diabetic human heart, cell death affects not only the myocyte compartment, but also endothelial cells (ECs) and smooth muscle cells (SMCs) of coronary vessels, potentially altering myocardial perfusion [3]. The loss of ECs and SMCs may lead to loss of arterioles and capillary structures, resulting in a rarefaction of the coronary circulation, an increase in minimal coronary vascular resistance, a reduction in coronary reserve, and defects in tissue oxygenation of the diabetic myocardium.

The diabetic state is extremely complex, and thereby difficult to reproduce in animal models.

Three metabolic alterations are present: hyperlipidemia, early hyperinsulinemia followed by pancreatic ß-cell failure, and hyperglycemia. IDDM differs from NIDDM because IDDM is not associated with a period of hyperinsulinemia and it is characterized by an early onset of hyperglycemia. Although the timing and severity of hyperglycemia may vary in IDDM and NIDDM, an increased blood glucose level is consistently found in both types of diabetes. For this reason, we have analyzed in this chapter the effects of hyperglycemia, RAS, and oxidative stress on the heart. The recognition that the adult heart possesses a pool of resident cardiac progenitor cells (CPCs), which are self-renewing, clonogenic, and multipotent [6, 7], dictates a different biologic understanding of cell regeneration in the heart. Diabetes may damage the CPC compartment, affecting the repair processes, and thereby cardiac function. Whether diabetes has consequences on CPCs comparable to those found in myocytes, ECs, and SMCs is an important unanswered question. If this were the case, diabetes may be viewed as a disease process that negatively affects the growth reserve of the heart so that enhanced cell death cannot be counteracted by a repopulation of the heart with cells that preserve the architecture and function of the myocardium.

Hyperglycemia, protein glycosylation, and diabetic cardiomyopathy

A relevant question with diabetes concerns the pattern of cell death and its consequences on the heart. Apoptosis is a rapid phenomenon, which allows sudden anatomical adaptation in response to abrupt changes in loading. The increase in diastolic stress is coupled with the immediate activation of apoptosis, side-to-side slippage of cells, mural thinning, and chamber dilation [8]. Apoptosis is very effective in removing undesired cells during development and the same may be true in the adult heart. Conversely, necrosis develops over a longer period and is not implicated in the precipitous restructuring of the wall, required for the preservation of stroke volume and cardiac output after infarction and other pathologic states [9]. Stretching of myocytes *in vitro* does not induce necrosis but triggers apoptosis, promoting an architectural rearrangement of the myocardium [10]. Apoptosis does not alter the architecture and composition of the myocardium, while cell necrosis results in an inflammatory reaction, vascular proliferation, and collagen deposition, leading to myocardial scarring. Although apoptosis and necrosis are two different biological processes, similar stimuli can initiate both forms of cell death. Ischemic insults induce myocyte apoptosis and necrosis, but the timing is different. The intensity of the death stimulus is responsible for the choice of the cell to die by either mechanism. This is the case for oxidative stress. Different levels of reactive oxygen species (ROS) trigger apoptosis or necrosis [11]. High formation of ROS can cause DNA damage together with covalent modifications of cellular proteins inducing double DNA strand breaks with blunt ends, typical of cell necrosis [12]. Oxidative stress increases exponentially with diabetes and is closely related to hyperglycemia, which is a common feature of both type I and type II diabetes.

Another biochemical complication with diabetes is the formation of glycosylated products [13]. Proteins constitute the principal substrate of this reaction, which generates glycoproteins in the extracellular compartment, plasma membrane, cytoplasm, and, ultimately, in the nucleus [14]. Glycosylation of proteins occurs through the addition of carbohydrates, with or without an enzymatic reaction [13]. Nonenzymatic glycosylation is commonly defined as glycation. The formation of glycation products requires time and becomes evident *in vitro* only when cells are kept in high glucose-containing media for at least 1 week [15]. The rate of glycation is slow and so rapidly turning-over intracellular proteins would not persist in the cell long enough to undergo nonenzymatic glycosylation. The most frequent product of enzymatic glycosylation is O-linked N-acetylglucosamine (GlcNAc). This posttranslational modification consists of single GlcNAc residues that are connected to the hydroxyl group of serine or threonine by a transferase catalyzing the O-glycosylation [14]. These sites are clustered at the COOH-terminal of intracellular proteins, in the proximity of proline or valine residues. They are similar to phosphorylation sites for several protein kinases [14].

Glycosylation and phosphorylation activate several transcription factors. The processes of glycosylation and phosphorylation are tightly and

dynamically regulated, and there is direct competition between glucose and phosphate at a single amino acid residue, resulting in a decrease in the level of phosphorylation when glycosylation occurs, and vice versa [16]. Glycosylation and phosphorylation activate SP1, AP-1, SRF, and p53. Enzymatic glycosylation of p53 has originally been documented in a carcinoma-derived cell line in which wild-type glycosylated p53 is constitutively expressed and is biologically active [17]. In these cancer cells, glycosylated p53 promotes transcription of target genes and activates apoptosis. Depending on the stretch of amino acids involved, posttranslational modifications of p53 have different effects on gene transcription. The basic region at the COOH-terminus of p53 represses DNA binding and maintains p53 in its latent form. This inhibition can be relieved by two mechanisms: by introduction of bulky groups, including sugars like GlcNAc, or by neutralization of charge through phosphorylation by protein kinase C, casein kinase II, and p38 MAP kinase [18]. The sites of glycosylation do not possess a common consensus sequence, but share some characteristics. They are clustered at the COOH-terminus in the proximity of proline and valine residues. They are located in sequences rich in serine and threonine that are similar to phosphorylation sites for protein kinases [16]. These sites constitute the substrate for the catalytic activity of glycosyl transferases and protein kinases.

The effects of hyperglycemia on the heart can be reproduced, at least in part, by culturing cells in the presence of concentrations of glucose that exceed the physiologic value of 5 mM. Binding sites for p53 are present in the promoter of angiotensinogen and Ang 1 receptor (AT_1) genes [8]. p53 enhances the myocyte RAS and the formation of Ang II. Moreover, p53 reduces the expression of genes opposing cell death, such as Bcl-2, and up-regulates genes promoting apoptosis, such as Bax. High glucose leads to O-glycosylation and activation of the p53 transcription factor. This posttranslational modification of p53 results in up-regulation of the cardiomyocyte RAS and synthesis of Ang II [19]. Ligand binding to AT_1 receptors in myocytes activates p38 MAP kinase that, in turn, phosphorylates p53 at Ser390, provoking a sustained stimulation of the transcription factor and chronic formation of Ang II (Figure 16.1). An additional site of phosphorylation of p53, Ser18, which is coupled with growth arrest and apoptosis, is activated when myocytes are cultured in the presence of high concentrations of glucose [19]. Ang II binding to AT_1 receptor increases cytosolic calcium, which in turn leads to ROS generation and ultimately cell death. This effector pathway is blocked by inhibitors of O-glycosylation. As expected, AT_1 receptor antagonists have no influence on O-glycosylation of p53 but interfere with p53 phosphorylation. Finally, inhibition of p38 MAP kinase mimics at a more distal level the consequences of losartan by preventing Ang II-mediated myocyte death [19].

It is reasonable to assume that CPCs possess all the molecular components of RAS, but the existence of this hormonal system in cardiac primitive cells has not been documented yet. If this were the case, the effects of Ang II on myocyte growth and death may similarly involve CPCs and the chronic up-regulation of RAS in diabetes may be associated with a decrease in the CPC pool size. Preliminary results are consistent with this possibility (Figure 16.2). Hyperglycemia may glycosylate

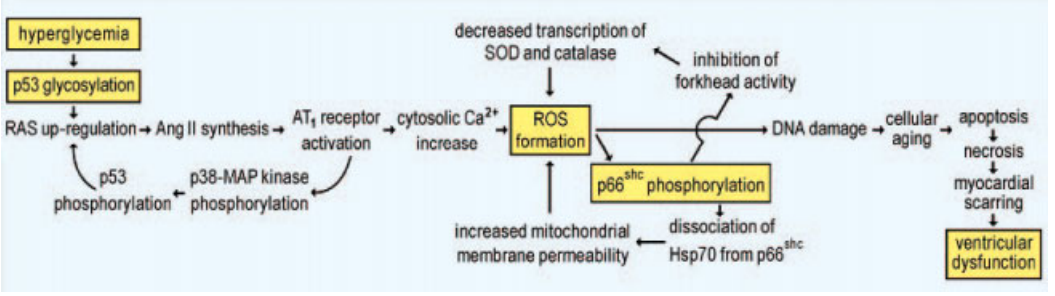


Figure 16.1 Central role of the renin–angiotensin system (RAS) in the generation of reactive oxygen species (ROS) and the development of the diabetic cardiomyopathy. SOD = superoxide dismutase.

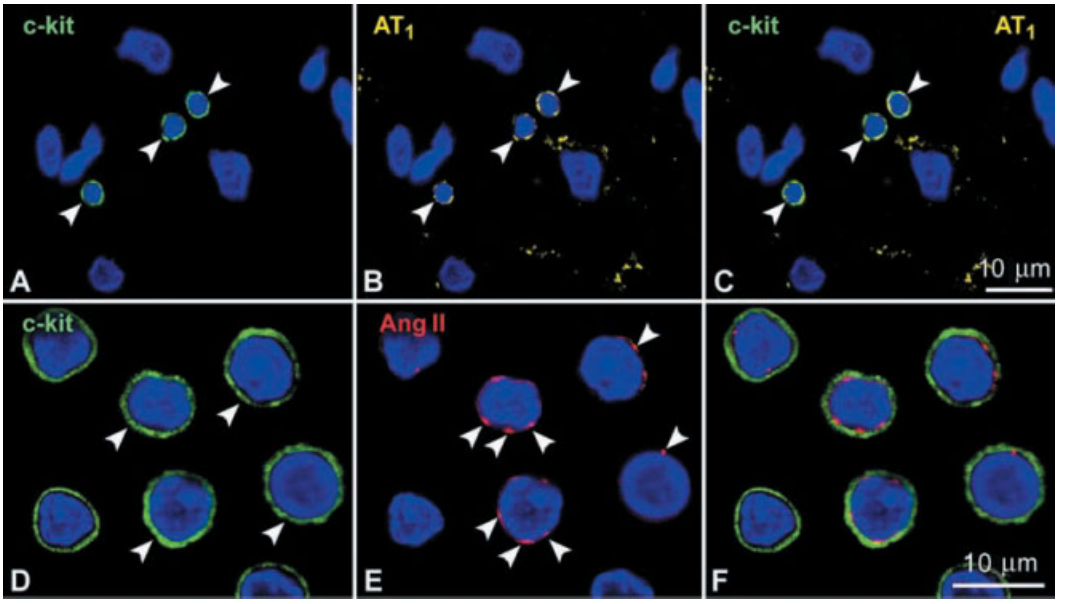


Figure 16.2 Components of the renin–angiotensin system are present in CPCs. (A–C) c-kit-positive (A; green, arrowheads) cells in the left ventricular myocardium express the AT_1 receptor subtype (B; yellow). Panel C represents a merge of panels A and B. (D–F) Isolated cardiac c-kit-positive (D; green, arrowheads) cells express in their cytoplasm Ang II (E; red, arrowheads). Panel F represents a merge of panels D and E.

p53 in CPCs, activating the cellular RAS and the synthesis of Ang II. Binding to AT_1 receptor and phosphorylation of p53 may stimulate chronically p53 function, transcription of p53-inducible genes, local Ang II production, reactive O_2, and CPC death. Perhaps, diabetic cardiomyopathy may be viewed as an Ang II-mediated and ROS-dependent myopathy in which aging and death of CPCs impair the normal turnover processes of myocytes, vascular ECs, and SMCs. This alteration may lead initially to a moderately dilated heart with ventricular dysfunction and chronically to a severely decompensated cardiomyopathy. If hyperglycemia and reactive O_2 are the determinants of the diabetic myopathy, similar mechanisms may be operative in both IDDM and NIDDM.

$p66^{shc}$ and the diabetic heart

The genetic–molecular status of the heart may influence the outcome of diabetes, and genetic manipulations may interfere with the development of a diabetic cardiomyopathy. The diabetic heart may benefit from a form of treatment in which cell therapy and gene therapy are combined. Cell transplantation alone may lead to an improvement of cardiac function that is only transient. The metabolic derangement characteristic of the diabetic state may negatively affect the survival and growth of the implanted CPCs. Genetic modifications aiming at the enhancement of cell viability by interfering with oxidative stress, apoptosis, and necrosis may confer a growth advantage to the exogenous cells with respect to resident cardiac cells. Moreover, the progeny of the delivered cells inherits the genetic background of the CPCs. The resistance of myocytes, SMCs, and ECs to the death stimuli is essential for the preservation of myocardial homeostasis. Based on evidence in the literature, it can be said that candidate genes can be identified in $p66^{shc}$ and insulin-like growth factor-1 (IGF-1).

The Shc family of proteins includes three splicing isoforms encoded by the same genetic locus—$p46^{shc}$, $p52^{shc}$, and $p66^{shc}$ [20]. $p46^{shc}$ and $p52^{shc}$ are ubiquitously expressed and possess a reciprocal relationship in every cell type. This is not the case for the $p66^{shc}$ isoform, which varies in abundance from cell to cell and it is not present in all cells. However, cells that do not possess $p66^{shc}$ in baseline conditions acquire the capacity to express

this protein in response to apoptotic stimuli [21]. $p46^{shc}$, $p52^{shc}$, and $p66^{shc}$ share a modular structure, which includes a C-terminal SH2 domain, a proline-rich CH1 domain, and a PTB domain, that interacts with tyrosine phosphoproteins and binds membrane phospholipids. The SH2 domain mediates the transmission of stimulatory growth signals from membrane tyrosine kinase receptors. $p46^{shc}$ and $p52^{shc}$ activate the Ras oncoprotein, and this interaction links Shc proteins to downstream effector responses involving MAP kinases and cell proliferation [20]. $p66^{shc}$ behaves in a different manner since it does not activate MAP kinases following growth factor stimulation. $p66^{shc}$ contains an additional N-terminal proline-rich CH2 domain that inhibits c-fos promoter activity [20], which differs from the role of the other Shc gene products. Thus, members of the same family of Shc proteins exert an opposite function: $p46^{shc}$ and $p52^{shc}$ promote cell proliferation, while $p66^{shc}$ negatively affects cell growth.

Ablation of $p66^{shc}$ only, without affecting $p46^{shc}$ and $p52^{shc}$, decreases the influence of oxidative stress *in vivo*. Targeted mutation of the mouse $p66^{shc}$ gene decreases the formation of ROS, increases the resistance of the cell to free radicals, and prolongs life by 30% [22]. By multiple pathways, p53 increases the intracellular concentration of ROS. The adaptor protein $p66^{shc}$ constitutes a powerful effector that links p53 to oxidative stress. The $p66^{shc}$ protein reduces the resistance of cells to ROS, stimulating the cell death pathway [22, 23]. The $p66^{shc-/-}$ mouse provides the first demonstration that a genetic link exists between reactive O_2 and cell death in mammals. Because of the high metabolic rate of the heart in mice, diabetes-mediated O_2 toxicity may affect the myocardium shortly after the onset of diabetes. The negative consequences of diabetes on the structure and function of the heart may be partially inhibited in $p66^{shc-/-}$ transgenic mice. This animal model offers the possibility to identify whether a genetic relationship exists between diabetes and ROS, on the one hand, and premature cellular senescence and heart failure, on the other.

$p66^{shc}$ transduces ROS-dependent signals only in the presence of p53. In the absence of p53, the ability of $p66^{shc}$ to modulate the elevation of intracellular oxidants and induce cell death is lost. In cells exposed to UV light or H_2O_2, the expression of $p66^{shc}$markedly increases [22]. However, this up-regulation does not take place in $p53^{-/-}$ cells, strongly suggesting that p53 positively modulates the adaptor protein $p66^{shc}$. These observations have been confirmed by the overexpression of p53 in $p53^{-/-}$ fibroblasts: this genetic manipulation restores the $p66^{shc}$ response [24]. By using ^{35}S metabolic labeling, it has been documented that p53 increases the stability of $p66^{shc}$. This phenomenon may be related to a posttranslational modification of $p66^{shc}$ by its phosphorylation at Ser36. Phosphorylation at Ser36 is critical for conferring to the cells an increased susceptibility to oxidative stress, and thereby initiating the cell death response with oxidative damage [21]. Although a synergistic interaction between p53 and $p66^{shc}$ has been demonstrated, the molecular mechanisms underlying this relation have not been clarified yet. It is known that c-Jun N-terminal protein kinase (JNK) is the enzyme that phosphorylates $p66^{shc}$ at Ser36 [25]. In the presence of high glucose levels, the up-regulation of RAS is coupled with an increased expression of JNK in cardiomyocytes [19]. p53 may act indirectly on $p66^{shc}$ by up-regulating RAS and, as a consequence, JNK and $p66^{shc}$ phosphorylation. JNK, in turn, increases p53 half-life by inhibiting its ubiquitination. Conversely, reduced JNK activity prevents cytochrome c release and caspase-3 cleavage. This inhibition of apoptosis could be mediated by the lack of JNK-mediated phosphorylation of $p66^{shc}$. Thus, Ang II, p53, and $p66^{shc}$ act in concert and create a positive feedback loop that leads to increased ROS formation, cell aging, and cell death. $p66^{shc}$ influences the role that p53 has in the activation of apoptosis with ROS formation but $p66^{shc}$ is not implicated in other functions of p53, including growth arrest and tumor suppression.

The N-terminal domain of $p66^{shc}$ becomes phosphorylated at Ser36 when cells are exposed to oxidative stress. Phosphorylated $p66^{shc}$ inhibits the activity of the forkhead family of transcription factors, leading to the reduced transcription of the forkhead target genes such as the antioxidants superoxide dismutase and catalase. Additionally, a fraction of intracellular $p66^{shc}$ is located within the mitochondrial compartment. In this organelle, $p66^{shc}$ binds to the heat shock protein Hsp70. This inert complex is activated and dissociated upon oxidative stress, leading to a marked decrease in mitochondrial membrane potential, an

increase in transmitochondrial permeability, and ROS formation [23]. For these reasons, wild-type and $p66^{shc-/-}$ mice can be employed to test the hypothesis that oxidative stress and cell death are the critical determinants of the diabetic heart. If this were correct, the impact of ROS on cardiac cells should be significantly attenuated in the $p66^{shc-/-}$ mouse, delaying the onset of a diabetic myopathy and heart failure.

High glucose concentration promotes the generation of ROS, and both apoptosis and necrosis have been found in CPCs from wild-type mice. However, CPCs from $p66^{shc-/-}$ mice are more resistant to hyperglycemia, which results in an attenuation of cell death. CPC death can only be opposed by defense mechanisms capable of decreasing the amount of ROS and/or attenuating the oxidative stress response. Ablation of $p66^{shc}$ may offer both forms of protection, since the production of ROS with hyperglycemia is reduced in $p66^{shc-/-}$ CPCs. Preliminary data *in vivo* suggest that a phenomenon comparable to hyperglycemia may be present with diabetes (Figure 16.3). Four weeks after the induction of diabetes with streptozotocin, CPC apoptosis is lower in diabetic $p66^{shc-/-}$ mice than in wild-type mice, and attenuation of cell death is accompanied by reduction in DNA oxidative damage and cellular senescence [26]. The generation of hydroxyl radicals, •OH, which was assessed by 8-OH-deoxyguanosine accumulation in nuclei, is blunted in $p66^{shc-/-}$CPCs. Similarly, the fraction of senescent $p16^{INK4a}$-positive CPCs is lower in diabetic $p66^{shc-/-}$ than in wild-type mice. There is a high association between DNA damage and $p16^{INK4a}$ expression, and apoptosis is restricted to $p16^{INK4a}$-positive CPCs in both wild-type and $p66^{shc-/-}$ mice. CPC necrosis is occasionally seen. Moreover, DNA damage, cellular senescence, and myocyte necrosis are blunted in diabetic $p66^{shc-/-}$ mice, while myocyte apoptosis is similar in diabetic wild-type mice and $p66^{shc-/-}$ mice. These data are consistent with the notion that oxidative stress is coupled with cellular senescence, and the age of the cell conditions activation of the programmed death sequence.

Ablation of $p66^{shc}$ in CPCs and myocytes may have dramatic beneficial consequences on the diabetic heart; $p66^{shc}$ deletion may decrease the extent of cell death and change cell death from necrosis to apoptosis. The CPC pool may be protected, and thereby myocyte regeneration and vessel formation can occur. If apoptosis rather than cell necrosis were the predominant outcome of diabetes in $p66^{shc-/-}$ mice, interstitial fibroblasts may not be activated and collagen accumulation, myocardial scarring, and cardiac remodeling may be largely avoided. In this regard, cardiac performance is moderately altered 1 week after the onset of diabetes and severe ventricular dysfunction is apparent at 4 weeks in wild-type mice. Conversely, cardiac performance is protected up to 4 weeks in diabetic $p66^{shc-/-}$ mice. Additionally, ventricular dilation is prevented and the myocyte number is preserved. These findings suggest that the impact of ROS on the heart is attenuated in $p66^{shc-/-}$ mice, delaying the onset of a diabetic myopathy. Therefore, hyperglycemia may result in p53 glycosylation, up-regulation of the local RAS, Ang II formation, p53 phosphorylation, further activation of RAS, $p66^{shc}$ phosphorylation, elevation in intracellular Ca^{2+}, production of reactive O_2, and, ultimately, cellular senescence and death. The attenuation of the effects of oxidative stress on the myocardium obtained with $p66^{shc}$ deletion appears to interfere with the development of a diabetic cardiomyopathy, in spite of pancreatic damage and hyperglycemia.

IGF-1 and the diabetic heart

Clinical and experimental studies have not provided a consistent answer regarding the identification of a therapeutic role for exogenously administrated IGF-1 or growth hormone in pathologic states of the heart [27, 28]. IGF-1 overexpression in transgenic mice has previously been shown to inhibit both myocyte apoptosis in the surviving myocardium after infarction [29] and myocyte necrosis after nonocclusive coronary artery constriction [30]. Interference with cell death improves cardiac anatomy and decreases diastolic wall stress in both situations. However, these beneficial effects are not accompanied by a corresponding amelioration in ventricular hemodynamics, possibly due to the presence of large infarcts and restriction in coronary perfusion, respectively. Therefore, the therapeutic impact of IGF-1 on the diseased heart remains unclear. In the diabetic heart, however, IGF-1 overexpression affects the level of activation of myocyte death and

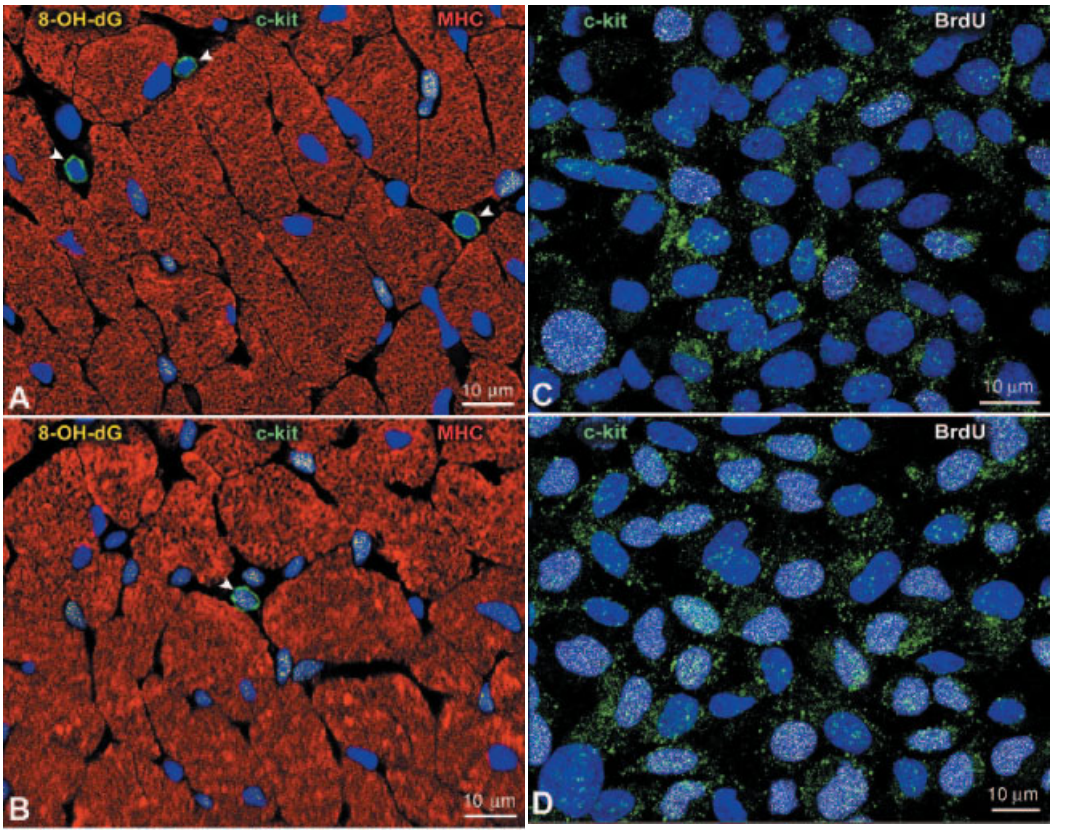


Figure 16.3 Oxidative damage and proliferation in CPCs. (A and B) Generation of 8-OH-deoxyguanosine (8-OH-dG) (yellow dots in the nuclei) in c-kit-positive CPCs (green; arrowheads) and myocytes (red; myosin heavy chain labeling) of control (A) and diabetic (B) mice. (C and D) BrdU incorporation (white dots in the nuclei) in clonogenic c-kit-positive (green) CPCs cultured in the absence (C) and in the presence (D) of IGF-1.

preserves ventricular performance (see also Chap 15). Myocyte death has been questioned as an etiological factor capable of inducing functional alterations. Cell death has been claimed to be an epiphenomenon that has little influence on the onset and evolution of cardiac failure. Recent data in diabetic nontransgenic and IGF-1 transgenic mice challenge this contention.

IGF-1 protects CPCs and myocytes from both apoptotic and necrotic cell death under a variety of pathologic conditions *in vivo* and *in vitro*. Importantly, IGF-1 interferes with the up-regulation of the cardiac RAS, attenuating the impact of Ang II on cell death. The decrease in Ang II synthesis induced by IGF-1 may limit the production of ROS, and thereby the activation of cell death mechanisms. Moreover, IGF-1 increases the expression of Bcl-2 and decreases the expression of Bax by affecting p53 stability and function. The enhanced quantity of Bcl-2 and the reduced amount of Bax in myocytes overexpressing IGF-1 provide additional bases for the antiapoptotic and antinecrotic effects of this growth factor. For these reasons, IGF-1 opposes or delays the development of diabetic cardiomyopathy. In the presence of IGF-1, ROS formation is only moderately increased and modest levels of myocyte apoptosis have been detected. Because of this negative influence of IGF-1 on p53 function, local RAS, Ang II production, oxidative stress, and myocyte death, no appreciable alterations in ventricular hemodynamics are found in IGF-1 transgenic mice at 7 and 30 days after streptozotocin administration [4]. IGF-1 does not interfere with p53 glycosylation but enhances the expression of the p53-inducible gene

Mdm2 by up-regulating Akt and Akt-dependent phosphorylation of the N-terminus of p53. The formation of p53-Mdm2 inactive complexes leads to a decreased synthesis of Ang II, reduced oxidative stress, and cell death. This mechanism of action of IGF-1 is operative chronically with diabetes, providing long-term beneficial effects on the diabetic heart.

The increase in local Ang II formation in the diabetic heart can be expected to increase cytosolic Ca^{2+} concentration. Defects in Ca^{2+} homeostasis occur in cardiac myocytes with diabetes, and nanomolar doses of Ang II have been shown to increase Ca^{2+}in these cells [19]. The elevation in intracellular Ca^{2+} shortly after Ang II stimulation is required for Ang II-mediated myocyte death. It is noteworthy that Ang II leads to the generation of ROS in myocytes, and this action is blocked by the Ca^{2+} chelator BAPTA/AM. Additionally, myocytes isolated from diabetic mice exhibit high levels of ROS, and this phenomenon is inhibited by losartan or tiron, documenting once more that Ang II and reactive O_2 are tightly linked in diabetes. It is unclear whether IGF-1 operates at the level of the mitochondria where ROS are generated. Without doubt, IGF-1 operates proximally at the level of the activation of p53 and Ang II synthesis [4]. By these actions, IGF-1 attenuates the induction of ROS with diabetes, protecting the heart from oxidative stress.

IGF-1 is mitogenic and antiapoptotic and is necessary for stem cell growth. CPCs possess IGF-1 receptors on their plasma membrane and are capable of synthesizing IGF-1. Thus, all the components of a cellular IGF-1/IGF-1 receptor system are present in CPCs, and IGF-1 protects CPC viability and enhances their growth in the intact and infarcted heart [31]. Importantly, IGF-1 opposes or delays the onset of the senescent phenotype in CPCs [32]. Diabetic cardiomyopathy may be viewed as a ROS-dependent myopathy in which cell loss initially produces moderate ventricular dysfunction and chronically leads to a severely decompensated heart. IGF-1 protects the myocardium from the detrimental effects of diabetes by attenuating Ang II synthesis and thus reactive O_2 damage and, ultimately, cell death. Whether the protective role of IGF-1 in diabetes persists over long periods remains an important unanswered question.

Conclusions

These observations point to the possibility that the imbalance between cardiac cell death and cell regeneration characterizes the diabetic heart. The death of mature myocytes, SMCs, and ECs is the initiating factor but the progression of the diabetic myopathy is mediated by premature cellular aging and the death of CPCs. The loss of CPCs favors cellular senescence and may promote a shift in the pattern of cell death from apoptosis to necrosis within the myocardium. Accumulation of old cells and cell necrosis alter the orderly organization of myocardial structure, depressing cardiac performance. Cellular senescence and attrition of the pool size of functionally competent CPCs lead to an insufficient replacement of old, dying cells and the acquisition of the heart senescent phenotype. Therefore, the diabetic cardiomyopathy can be viewed as a stem cell myopathy in which a defective stem cell compartment causes premature aging and the death of myocytes, SMCs, and ECs.

Acknowledgment

This work was supported by NIH.

References

1 Fein FS, Sonnenblick EH. Diabetic cardiomyopathy. Prog Cardiovasc Dis 1985; 27:255–270.
2 Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol 2003; 65:45–79.
3 Frustaci A, Kajstura J, Chimenti C, et al. Myocardial cell death in human diabetes. Circ Res 2000; 87:1123–1132.
4 Kajstura J, Fiordaliso F, Andreoli AM, et al. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. Diabetes 2001; 50:1414–1424.
5 Heart Outcomes Prevention Evaluation (HOPE) Study Investigators. Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-Hope substudy. Lancet 2000; 355:253–259.
6 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.
7 Linke A, Muller P, Nurzynska D, et al. Stem cells in the dog heart are self-renewing clonogenic, and multipotent and regenerate infracted myocardium, improving cardiac function. Proc Natl Acad Sci USA 2005; 102:8966–8971.

8 Anversa P, Olivetti G. Cellular basis of physiologic and pathologic myocardial growth. In Page E, Fozzard HA, Solaro RJ (eds): Handbook of Physiology, Section 2: The Cardiovascular System: The Heart, Vol. 1. Oxford: Oxford University Press, 2002; 75–144.

9 Anversa P, Nadal-Ginard B. Myocyte renewal and ventricular remodelling. Nature 2002; 415:240–243.

10 Cheng W, Li B, Kajstura J, et al. Stretch-induced programmed myocyte cell death. J Clin Invest 1995; 96:2247–2259.

11 Jacobson MD. Reactive oxygen species and programmed cell death. Trends Biochem Sci 1996; 21:83–86.

12 Didenko VV, Hornsby PJ. Presence of double-strand breaks with single-base 3′ overhangs in cells undergoing apoptosis but not necrosis. J Cell Biol 2006; 135:1369–1376.

13 Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetes complications. N Engl J Med 1988; 318:1315–1321.

14 Haltiwanger RS, Busby S, Grove K, et al. O-glycosylation of nuclear and cytoplasmic proteins: regulation analogous to phosphorylation? Biochem Biophys Res Commun 1997; 231:237–242.

15 Giardino I, Edelstein D, Brownlee M. Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity. J Clin Invest 1994; 94:110–117.

16 Hart GW, Greis KD, Dong LY, et al. O-linked N-acetylglucosamine: the "yin-yang" of ser/thr phosphorylation? Adv Exp Med Biol 1995; 376:115–123.

17 Shaw P, Freeman J, Bovey R, Iggo R. Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. Oncogene 1996; 12:921–930.

18 Ashcroft M, Kubbutat MHG, Vousden KH. Regulation of p53 function and stability by phosphorylation. Mol Cell Biol 1999; 19:1751–1758.

19 Fiordaliso F, Leri A, Cesselli D, et al. Hyperglycemia activates p53 and p53-regulated genes leading to myocyte cell death. Diabetes 2001; 50:2363–2375.

20 Migliaccio E, Mele S, Salcini AE, et al. Opposite effects of the $p52^{shc}$/$p46^{shc}$ splicing isoforms on the EGF receptor-MAP kinase-fos signaling pathway. EMBO J 1997; 16:706–716.

21 Pacini S, Pellegrini M, Migliaccio E, et al. p66SHC promotes apoptosis and antagonizes mitogenic signaling in T cells. Mol Cell Biol 2004; 24:1747–1757.

22 Migliaccio E, Giorgio M, Mele S, et al. The $p66^{shc}$ adaptor protein controls oxidative stress response and life span in mammals. Nature 1999; 402:309–313.

23 Giorgio M, Migliaccio E, Orsini F, et al. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 2005; 122:221–233.

24 Pelicci G, Dente L, De Giuseppe A, et al. A family of Shc related proteins with conserved PTB, CH1 and SH2 regions. Oncogene 1996; 13:633–641.

25 Ravichandran KS. Signaling via Shc family adapter proteins. Oncogene 2001; 20:6322–6330.

26 Cascapera S, Rota M, Padin-Iruegas ME, et al. Diabetic Cardiomyopathy is a cardiac stem cell disease. Circulation 2005; 112(suppl II):ii–226.

27 Fazio S, Sabatini D, Capaldo B, et al. A preliminary study of growth hormone in the treatment of dilated cardiomyopathy. N Engl J Med 1996; 334:809–814.

28 Osterziel KJ, Strohm O, Schiler J, et al. Randomised, double-blind, placebo-controlled trial of human recombinant growth hormone in patients with chronic heart failure due to dilated cardiomyopathy. Lancet 1998; 351:1233–1237.

29 Li Q, Li B, Wang X, et al. Overexpression of insulinlike growth factor1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. J Clin Invest 1997; 100:1991-1999.

30 Li B, Setoguchi M, Wang X, et al. Insulin-like growth factor-1 attenuates the detrimental impact of non-occlusive coronary artery constriction on the heart. Circ Res 1999; 84:1007–1019.

31 Urbanek K, Rota M, Cascapera S, et al. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res 2005; 97:663–673.

32 Torella D, Rota M, Nurzynska D, et al. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 2004; 94:514–524.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

17 CHAPTER 17

Stem cells and cardiac aging

Roberto Bolli, MD, *& Piero Anversa,* MD

Introduction

This chapter addresses the interaction between cardiac aging and ischemic heart disease. Cardiac aging and myocardial infarction have been chosen as primary targets because myocardial infarction is a frequent occurrence in the elderly and one of the major causes of death in this population. Additionally, aging and ischemic cardiomyopathy are characterized by forms of structural damage that are common to almost all cardiac diseases. The recognition that the mammalian heart contains primitive undifferentiated cells with the properties of stem cells suggests that novel strategies may be developed to interfere with the detrimental effects of aging alone and aging and ischemic injury together. The identification of a pluripotent cardiac stem cell (CSC) impels a reevaluation of the possible interventions that can repair the myocardium damaged by a physiological stress, such as age, a pathological stress, such as infarction, or their combination. Myocardial infarction consists of a segmental loss of ventricular tissue that leads to scarring of a significant portion of the wall. Advanced age is typically associated with multiple sites of injury, which appear as areas of myocardial loss in various phases of healing, scattered throughout the ventricular wall. These two aspects of cardiac damage coexist in ischemic cardiomyopathy, while foci of reparative fibrosis and interstitial fibrosis prevail in the aging heart.

A relevant question is whether myocardial regeneration by CSCs can replace large amounts of scarred tissue, leading to reverse remodeling of the aged infarcted heart. Old CSCs may be less efficient than young cells, markedly affecting the growth reserve of the senescent heart. However, residual CSC replication and differentiation could enhance myocyte turnover and replace old, enlarged, poorly contracting cells with smaller, more numerous younger myocytes. Thus, this remaining pool of functionally competent primitive cells might be capable of restoring partly infarcted myocardium. Cell renewal is expected to include both smooth muscle cells and endothelial cells generating younger coronary vessels. However, the actual degree of myocardial repair that can be accomplished in the old decompensated heart is currently unknown. The old heart is faced with growth limitations, and the structural outcome would be an increase in myocyte heterogeneity with a larger population of markedly hypertrophied senescent myocytes and a smaller population of middle-aged and young, newly formed cells. A comparable phenomenon would be expected to involve the various segments of the coronary vasculature with a decrease in the number of resistance arterioles and capillary profiles.

Although this may be seen as an oversimplification of a very complex problem, this notion is consistent with the view that myocardial aging results from replicative senescence of CSCs. The assumption that growth arrest of CSCs is equivalent to cardiac senescence provides a unique and novel parameter that can be measured to assess the consequences of aging alone and age and ischemic injury together on the heart. Importantly, this approach may allow us to identify premature or delayed myocardial aging. An early entry of CSCs into a permanently quiescent state would produce precocious senescence of myocytes, vascular smooth muscle cells and endothelial cells. Apoptotic death is enhanced in old cells and this factor, coupled with inadequate cell regeneration, would contribute to ventricular dysfunction. Conversely, late acquisition of growth

arrest by CSCs would delay cardiac aging and preserve cardiac function.

The aging heart

Although myocardial aging in humans has been studied extensively, the possibility that an aging cardiomypathy can develop in the elderly remains a controversial concept [1]. The selection of patients has always been based on the chronological age of otherwise healthy individuals, paying little attention to the fact that chronological age and physical age do not necessarily coincide, and organism and organ age do not proceed at the same pace [2]. In general, there is little appreciation for these unpredictable factors, and currently aged patients with cardiac decompensation are diagnosed according to classified diseases, excluding the possibility that aging per se can be the etiology of the pathological condition. In this regard, efforts have been made to determine whether aging alone results in a dilated myopathy with characteristics different from idiopathic dilated cardiomyopathy (IDC). Several criteria have been used to distinguish these two disease states [3]. The aged decompensated heart demonstrates a moderate increase in ventricular mass; however, the thickness of the posterior wall of the left ventricle and interventricular septum is within the normal range. Conversely, severe hypertrophy is detected with IDC (Table 17.1). Additionally, the aging myopathy becomes apparent in individuals 70 years old and older, an age unusual for IDC. Ventricular dilation and overt left and right ventricular failure are present in both groups of patients. However, coronary angiography is normal and valvular abnormalities are absent. The diagnosis of IDC and aging myopathy requires that the syndrome not be explained by hypertension, ischemic heart disease, diabetes mellitus, systemic disorders, or a history of drug or alcohol abuse. Observations from animal studies are consistent with these human findings; aging in rodents typically produces a dilated myopathy that develops over time, leading to a progressive alteration in cardiac performance [4]. Thus, myocardial aging differs from IDC; it develops at an older age and shows modest cardiac hypertrophy, supporting the notion of an independent form of cardiac disease in the elderly.

The changes in the size and shape of the old heart are coupled with modifications in the volume composition of the myocardium and the number of cardiomyocytes within the ventricular wall. Early after birth, during the transition from postnatal life to adulthood, there is a well-coordinated growth adaptation of myocytes, the coronary vasculature, and the microvasculature, including the capillary network. This proportional physiological growth is lost in pathologic hypertrophy, which is characterized by sustained pressure or volume overload on the heart. A similar abnormality develops in the aging myocardium, mimicking decompensated ventricular hypertrophy [5]. Previous studies in humans have provided convincing evidence in men, but surprising results in women [6]. The loss of myocytes, arterioles, and capillaries was noted in the old male heart in both humans and animals [5], but not in

Table 17.1 Aging myopathy.

	Control hearts	*Aging myopathy*	*IDC*	*Normal range*
Age (years)	76 ± 4	73 ± 2	61 ± 4	
Duration of disease (months)	N/A	20 ± 9	90 ± 61	
LV weight (g)	155 ± 28	277 ± 53	394 ± 116	140–210
LVPT (mm)	9.3 ± 0.8	9.8 ± 1.2	11.3 ± 2.0	6–11
IVST (mm)	9.7 ± 0.8	10.4 ± 1.8	12.4 ± 0.23	6–11
LVEDD (mm)	47 ± 4	63 ± 7	75 ± 11	34–52
Ejection fraction (%)	57 ± 3	27 ± 6	24 ± 4	>50

IDC = idiopathic dilated cardiomyopathy; LV = left ventricle; LVPT = left ventricular posterior wall thickness; IVST = interventricular septum thickness; LVEDD = left ventricular end-diastolic diameter; N/A = not applicable.

young, middle-aged, or old women. New findings, however, suggest that a similar phenomenon occurs in the senescent female heart (Anversa P unpublished results). However, myocyte death takes place in women very late in life. Relative to men, the process in women is delayed by nearly 10 years.

The mechanisms responsible for the development of the aging myopathy in humans are currently unknown. Myocyte apoptosis and necrosis are evident in young mice and rats and increase with age [5, 7]. Foci of replacement fibrosis and diffuse interstitial fibrosis are frequent and affect mostly the inner layer of the left ventricle, although they are also present in the midmyocardium and epimyocardium. Myocyte hypertrophy and replication tend to preserve muscle mass and function, which ultimately deteriorates in the old animals [8]. With time, myocyte division becomes the prevailing form of reactive growth, while myocyte hypertrophy declines [9, 10]. Myocyte multiplication, however, is not sufficient to sustain an appropriate cell number. Subsequent cavitary dilation, with a decreased ventricular mass-to-chamber volume ratio, an elevation in wall stress, and impairment of the mechanical behavior of myocytes become apparent, and cardiac failure supervenes.

Aging and myocardial infarction

Coronary artery disease and age are two of the major causes of morbidity and mortality in the Western world and in the United States in particular. Myocardial infarction is a frequent event in the old population where it has been shown to have more devastating consequences than those seen in young individuals [11]. Recently, we have documented that myocyte regeneration takes place immediately after an acute infarction in humans, but is restricted to the infarct border zone and the distant viable tissue, occasionally invading the dead myocardium [12]. This form of cell growth does not interfere with the healing process, scar formation, and the development of an ischemic myopathy. Similarly, in the old heart, myocyte multiplication occurs in proximity to areas of tissue damage or in the intact healthy myocardium. Myocyte proliferation is not sufficient to replace the foci of collagen accumulation or to prevent cavitary dilation and the decline in pump performance of the senescent heart.

Over the last two decades, the origin of the multiplying myocytes had remained a major unresolved problem, but the recent recognition of primitive and progenitor cells in the adult human heart under normal conditions as well as in the pressure overloaded, aged, and infarcted human heart has shed some light on the possible source of these growing cells [13]. CSCs may undergo lineage commitment and during division progressively acquire structural proteins reaching functional competence. The accumulation of contractile and noncontractile elements within the cytoplasm leads to the adult myocyte phenotype and growth arrest. Multiplication is no longer possible, and these myocytes can experience only cellular hypertrophy. However, a group of young, relatively small cells does not reach complete differentiation, retaining the ability to undergo a finite number of divisions. The identification of telomerase in small cycling myocytes points to the presence of amplifying cells developed from CSCs. Amplifying myocytes may constitute a growth reserve of the heart, set aside for use in time of need. In emergency situations, these cells can reenter the cell cycle, rapidly replicate, and significantly increase the number of working myocytes. These growth processes are limited mostly to the viable myocardium.

To repair the acutely infarcted aged heart, it is necessary to address three distinct types of structural damage: (1) the infarct, in which cells are apoptotic and/or necrotic; (2) the noninfarcted old ventricle, in which there are foci of scarred tissue and areas of injury in various phases of healing; and (3) diffuse interstitial fibrosis. Chronically, the infarcted region is replaced by a large scar, which would make reconstitution of the ventricle more difficult. The old, spared myocardium possesses the same characteristics indicated in points 2 and 3. The question then concerns the modality of intervention, the cells to be employed for cardiac repair, the ability of these cells to grow and differentiate, and their long-term destiny. These are critical issues because the cells used so far in a variety of experiments have not been proven to become adult myocytes [14]. Similarly, the lifespan of newly formed cells in the regenerating myocardium is unknown. Moreover, the induction of angiogenesis is as important as myocyte replication. Successful repair requires reconstitution of the myocardium and, therefore, new parenchymal

cells and coronary vessels, which are functionally competent and express the adult phenotype.

Primitive and early committed cells accumulate acutely in the region bordering the infarct [12]. After homing, these cells grow and differentiate in new myocytes and coronary vessels. However, a block exists at the sharply defined boundary that separates the viable myocardium of the border zone from the dead tissue of the infarct. CSCs, progenitors, and precursors do not cross this boundary, and thus their translocation to the dead myocardium is impeded. Such an obstruction hampers the reconstitution of infarcted myocardium and the recovery of function. This phenomenon is of great clinical relevance because it is also operative in other infarcted organs including the liver, skin, brain, kidney, and testis [13]. Consequently, therapeutic strategies need to be directed to the activation of exogenously administered or resident primitive cells to facilitate their survival, movement, and successful competition with the inflammatory cells and fibroblasts present within the infarct. If these approaches were effective, the healing process could be modified. Ultimately, the dead tissue might be replaced by a newly formed functioning myocardium.

Aging, oxidative stress, and cell death

A common aspect of both the chronically infarcted heart and myocardial aging is apoptotic death of CSCs [3, 12], which affects the growth reserve of the myocardium and its ability to expand and counteract the abnormal increase in mechanical load. Observations in animals and humans strongly suggest that alterations in the formation of reactive oxygen species (ROS) may play an important role in the progression of the aging myopathy and the terminal evolution of the old infarcted heart. Aging and/or pathological stresses result in an exponential increase in oxidative stress [15], and different levels of ROS trigger apoptosis and necrosis [16]. ROS can cause cumulative damage to proteins, lipids, and nucleic acids, particularly in cells with regenerative potential, a typical characteristic of CSCs. DNA oxidation leads to single and double DNA strand breaks [3, 12, 17].

Oxidative injury is inherent in the atomic structure of O_2. By single electron additions, molecular oxygen sequentially generates superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$). $O_2^{\bullet -}$ and H_2O_2 are only moderately reactive with other molecules, but $^{\bullet}OH$ is highly reactive and causes extensive oxidative damage to macromolecules. $^{\bullet}OH$ can be responsible for most of the damage induced by ROS in biological systems [18]. Oxidative stress is also dependent on the interaction of nitric oxide (NO), generated in the endothelium, with $O_2^{\bullet -}$ and the formation of peroxynitrite $(ONOO)^{\bullet -}$ [19]. $ONOO^-$ may decompose into an oxidant with reactivity similar to $^{\bullet}OH$. Depending on the levels of $O_2^{\bullet -}$, NO production may decrease or enhance cell death in the vessel wall [20], adjacent myocytes [21], fibroblasts, or CSCs [3, 12].

Probes are available for the identification of H_2O_2 and $^{\bullet}OH$ together and $O_2^{\bullet -}$ in living cells. However, in tissue sections the analysis of H_2O_2-$^{\bullet}OH$ and $O_2^{\bullet -}$ is impossible. It is also impossible to measure $ONOO^-$ in individual cells or tissue samples. However, $ONOO^-$ interacts with proteins forming nitrotyrosine, which can be detected on a cellular basis. There is no documentation yet that $ONOO^-$ leads to DNA lesions and cell death, but results supporting this interaction have been obtained both in the failing dog heart [22] and in myocardial biopsies of diabetic patients [23]. The percentage of cardiac cells exhibiting nitrotyrosine increases in the canine decompensated heart and in the diabetic human heart, and this modified amino acid is closely linked with cell apoptosis. Additionally, the impact of ROS on DNA damage can be identified in tissue sections by the 8-OH-deoxyguanosine (8-OH-dG) probe, which recognizes $O_2^{\bullet -}$-mediated nucleotide injury. This type of DNA damage has been demonstrated in the myocardium of patients after acute and chronic infarcts [12].

DNA is extremely sensitive to ROS-induced damage. Of the four DNA bases, guanine is the most sensitive to ROS-induced damage. The hydroxylation of the C-8 position of 2′-deoxyguanosine results in the formation of 8-OH-dG, which is the most prominent form of free radical-induced DNA injury [24]. Other dG modifications having significant effects on DNA may occur as well; they include formamidopyrimidine, imidazole, oxaluric acid, oxalozone, cyanuric acid, guanidinohydantoin, spiroiminodihydantoin 1, and spiroiminodihydantoin 2. While accumulating $^{\bullet}OH$ attacks DNA, leading to the formation of 8-OH-dG, other forms

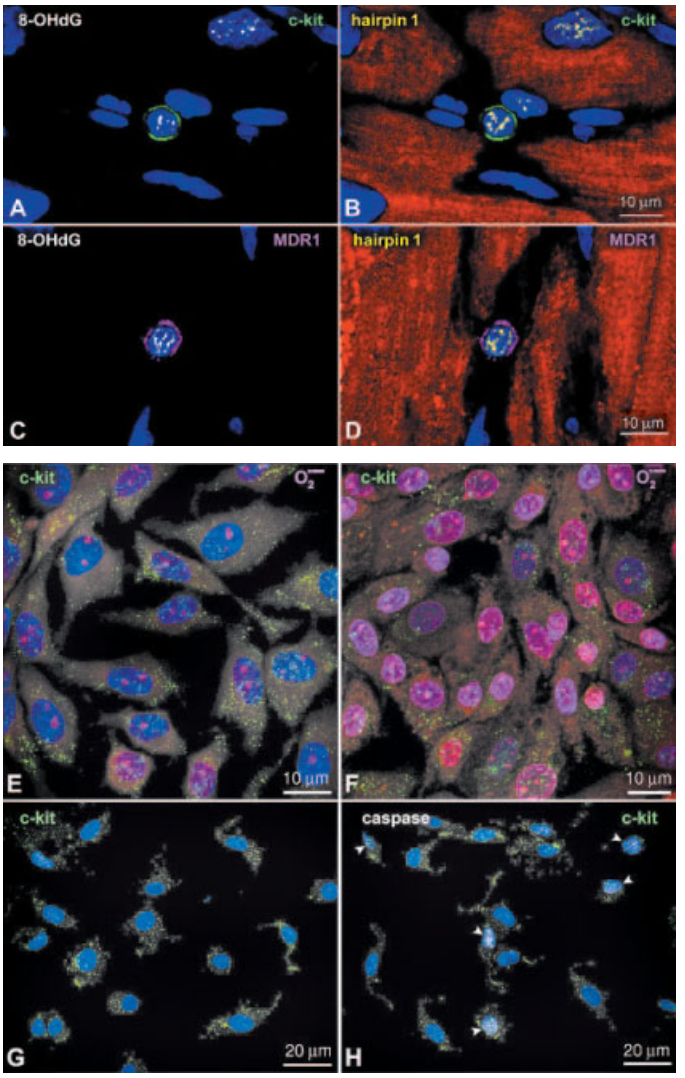


Figure 17.1 ROS and CSC apoptosis. (A–D) 8-OH-dG probe (A and C; white dots) was detected in c-kit- (A and B; green) and MDR1- (C and D; magenta) positive cells in the human myocardium in combination with apoptosis (B and D; hairpin 1, yellow dots). (E and F) Clonogenic c-kit-positive rat CSCs were cultured in the presence of low Ca^{2+} (E; 0.3 mM) and high Ca^{2+} (F; 3 mM) concentration. The formation of superoxide anion in mitochondria was detected by MitoSox Red (shown by magenta fluorescence). The signal of the oxidized probe in the cytoplasm and nuclei was markedly increased in the presence of high Ca^{2+} levels. Nuclei are shown by Hoechst 33432 (blue). (G and H) Clonogenic c-kit-positive (green) rat CSCs were cultured in the presence (H) and absence (G) of H_2O_2 (1 mM). Oxidative stress resulted in the activation of caspase-3 (H; white, arrowheads). Nuclei are shown by propidium iodide (blue).

of ROS are also implicated. Lipid hydroperoxides or lipid alkoxyl radicals contribute to the generation of 8-OH-dG under glutathione depletion [25]. The DNA repair machinery is able to excise these oxidized bases in the genomic DNA, but prolonged oxidative insults will cause it to accumulate. Thus, the presence of 8-OH-dG is a sensitive marker of ROS-induced DNA damage both *in vitro* and *in vivo*; notably, 8-OH-dG has been detected in c-kit- and MDR1-positive cells of the human myocardium together with apoptosis (Figure 17.1).

Mitochondria constitute the cellular site with the highest formation of ROS. Mitochondria reduce O_2 to H_2O via a 4-electron transfer by cytochrome *c* oxidase. Approximately 2–4% of the total O_2 consumed is not reduced to H_2O but to $O_2^{\bullet-}$ by the iron–sulfur proteins and by coenzyme Q. The rate of mitochondrial respiration and, therefore, the rate of $O_2^{\bullet-}$ generation are modulated by the coupling state of mitochondria. An increase in cytosolic Ca^{2+} leads to an excess uptake of this cation, enhancing respiration, oxygen utilization, and $O_2^{\bullet-}$ generation. Ca^{2+} loaded mitochondria produce H_2O_2 and lipid peroxides. Alterations in Ca^{2+} and mitochondrial respiration occur with aging [26], potentiating the production of $O_2^{\bullet-}$ and H_2O_2. Cytochrome *c* oxidase decreases and this correlates with the concomitant increase in the flux of mitochondrial $O_2^{\bullet-}$ and H_2O_2.

Similarly, antioxidant enzymes decrease in the aging heart. The mitochondrial function of primitive cells in young and old myocardium is unknown, but aged CSCs may have alterations of their mitochondrial compartment and enhanced susceptibility to death stimuli (Figure 17.1).

Cell death initiated by oxidative stress has been suggested to be the cause of the development of the aging myopathy. DNA damage generated by ROS may stimulate the autoproteolytic cleavage of p53. With double strand DNA breaks, N-terminal cleavage of p53 takes place and p50 (ΔN) is formed. p50 (ΔN) activates p53 target genes and promotes apoptosis. Conversely, cleavage of the C-terminus of p53 is coupled with single DNA strand breaks and the formation of p50 (ΔC). This fragment of p53 favors growth arrest in G1 and DNA repair [27]. The C-terminus of p53 is critical for the onset of apoptosis; its removal attenuates the apoptotic effect of this tumor suppressor gene [22, 28]. Additionally, p50 (ΔN) is not inhibited by Mdm2, while p50 (ΔC) can form p50 (ΔC)-Mdm2 inactive complexes. p53 might be directly involved in the initiation of irreversible DNA damage and death of CSCs. p50 (ΔC) and p50 (ΔN) proteins are apparent in primitive cardiac cells collected from old animals; p50 (ΔN) is higher than p50 (ΔC). High levels of ROS have been implicated in cell necrosis [16]. This form of cell death does not require the cleavage of p53 and the effects of p53 fragments on DNA strand breaks (Figure 17.2). The magnitude of oxidative stress may be so large that membrane integrity is lost, mitochondrial function is impaired, lysosomal proteases and nucleases are released, and nucleosomal and internucleosomal DNA are damaged.

Apoptosis involves the activation of caspases, which are aspartic acid specific cysteine proteases. They are present as inactive proenzymes and their activation requires proteolytic cleavage. Two major pathways of apoptosis have been identified [29]. The extrinsic pathway is triggered by proteins containing death domains, such as TNF and Fas, which interact on the cell surface with death effector domains, such as receptor-like structures. In the intrinsic pathway, cytochrome c, released from the intermembrane space of the mitochondria, binds the caspase-activating protein Apaf-1, which, in turn, stimulates procaspase-9, promoting its activation. Once activated, caspase-9 cleaves and activates procaspase-3

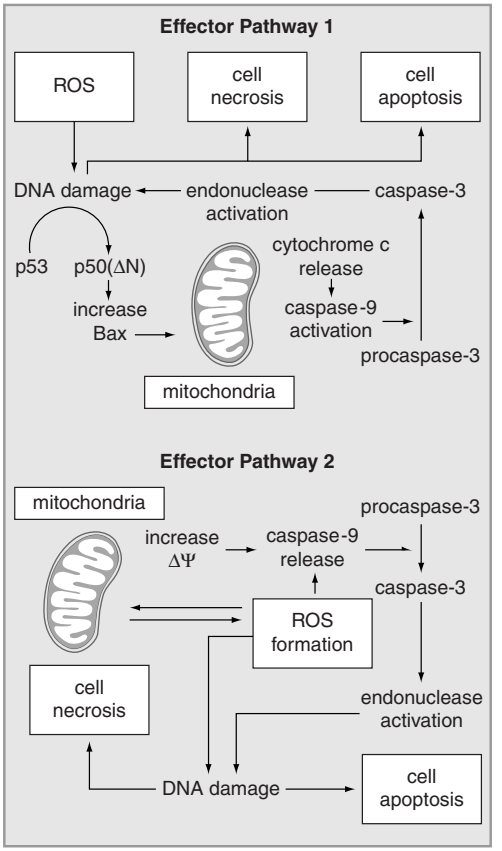


Figure 17.2 Apoptosis effector pathways.

(Figure 17.1), resulting in a cascade of additional caspase activation. Caspase-9 is the apical caspase in the cytochrome c pathway for apoptosis and is located in the intermembranous compartment of mitochondria of several cells including myocytes. The unique localization of this enzyme suggests that loss of mitochondrial barrier function needs to occur for this caspase to have access to its substrate [29]. Bcl-2 and Bcl-x_L can inhibit cytochrome c release from the mitochondria while Bax can promote its release.

p50 (ΔN) can up-regulate Bax and increase mitochondrial permeability, which promotes the release of cytochrome c. However, caspase-3 activation contributes only to the final disassembly of the cell. Alternatively, ROS may alter mitochondrial membrane potential (ΔΨ). The increase in ΔΨ is an early event of apoptosis. Cells with high ΔΨ are committed to die whereas cells with low ΔΨ have the ability to escape from apoptosis [30]. ΔΨ can

activate caspase-3 via a mechanism that is currently unknown but could involve the release of caspase-9, independently from cytochrome c [31]. The main difference between these pathways is the distinct role played by caspases in the apoptotic process. In pathway 1, caspases are secondary events, while in pathway 2, caspases play a critical role in cell death (Figure 17.2). Results obtained in myocytes from com pensated and decompensated hearts suggest that the effector pathway 1 prevails in the failing heart, where cytochrome c release and both procaspase-9 and procaspase-3 activities are increased [22].

In humans with a premature aging myopathy, CSC death by apoptosis is significantly increased [3]. In these patients, apoptosis of c-kit-positive cells is coupled with the expression of $p16^{INK4a}$, which is an established marker of cellular senescence [32]. $p16^{INK4a}$ inhibits cyclin-dependent kinase-4 and -6, opposing the reentry of cells into the cell cycle by inducing their arrest in early G0–G1 [32]. This form of growth arrest is irreversible since $p16^{INK4a}$ forms highly stable binary complexes with cyclin-dependent kinases. This is not the case when cells are blocked in G1 by $p21^{Cip1}$. This transient growth arrest allows the cells to repair the DNA and progress through the cell cycle. This is made possible by the physical characteristics of the p21-cyclin-dependent kinase complex, which can be easily dissociated by nuclear accumulation of cyclins. Typically, aging fibroblasts are stopped in G0–G1 by transient expression of $p21^{Cip1}$, followed by a temporary up-regulation of $p27^{Kip1}$ and then by persistent elevation of $p16^{INK4a}$. A similar phenomenon occurs in aging mouse myocytes in which the progressive decrease of $p21^{Cip1}$ expression is coupled with increased quantity of $p16^{INK4a}$ and $p27^{Kip1}$ [33]. Together, these observations point to a link between ROS, on the one hand, and cellular aging and death, on the other.

Cellular senescence

Replicative senescence describes the phenotype of cells that have experienced multiple rounds of division and have reached growth arrest. The Hayflick limit is dictated by specific changes in specialized cell cycle proteins [34]. The difficulty in the application of this *in vitro* finding to the *in vivo* condition has led to a heated controversy, and questions have been raised concerning the validity of this biological process. The possibility that the loss of proliferative capacity may result from "culture shock" and not from a "mitotic clock" has been suggested and the notion of replicative senescence has been challenged [35]. Confusion in the field was potentiated by conflicting results obtained from the studies of dermal fibroblasts and epidermal keratinocytes *in vivo* [36]. Although a progressive accumulation of senescent cells occurs in the skin of old individuals, cutaneous biopsies collected from the same patients at different ages failed to confirm the original observation [37].

A major obstacle encountered in the assessment of cellular senescence *in vitro* and *in vivo* relates to the selection of specific biomarkers of cellular aging. In fact, senescent cells cannot be distinguished from quiescent or terminally differentiated cells arrested in G1. One of the earliest biologic markers employed for the *in vivo* evaluation of replicative senescence involved the cytoplasmic accumulation of beta-galactosidase (β-Gal), which is detected at a pH of 6 [38]. This enzyme is present in senescent fibroblasts and keratinocytes, but is not found in quiescent fibroblasts and terminally differentiated keratinocytes. It is also absent in immortal cells but is induced by genetic manipulations that reverse immortality. Moreover, an age-dependent increase in senescence-associated β-Gal (SA-β-Gal) is observed in dermal fibroblasts and epidermal keratinocytes in humans [38]. However, recent reports have shown that SA-β-Gal increases in low-density cultures of senescent cells as a function of *in vitro* replicative age, not donor age [37]. The level of histochemically detectable SA-β-Gal is also elevated in immortal fibroblasts at high cell density and in low-density cultures of young cells exposed to H_2O_2. Thus, SA-β-Gal may be an indicator of cellular stress more than senescence.

To date, the most reliable marker of cellular senescence is the modifications of the telomerase–telomere axis together with the expression of the cell cycle inhibitors $p16^{INK4a}$ and p53 [39]. Telomerase activity is present in the normal adult human heart and is increased in myocardial hypertrophy [40] and premature myocardial aging [3]. This ribonucleoprotein, however, does not necessarily prevent telomere erosion; severe telomeric shortening has been detected in both myocytes

and CSCs [3, 41]. These observations are consistent with results obtained in hematopoietic stem cells (HSCs). HSCs express low to moderate levels of telomerase, but telomeres shorten considerably with age [42]. Two possibilities have been proposed in an attempt to explain telomeric shortening in the presence of detectable telomerase activity: a suppressor of telomerase function may be stimulated during the cell cycle or a competition may occur between the reassembly of telomeric chromatin and the extension of telomeres by telomerase [42]. In HSCs, telomere shortening occurs at a rate of 50–100 base pairs per population doublings (bp/pd), which is only slightly lower than the 50–150 bp/pd rate, characteristic of telomerase-negative cells [43]. It is reasonable to assume that the aging effects on CSCs lead to an imbalance between telomerase activity and length of telomeres, resulting in critical telomeric shortening, permanent withdrawal from the cell cycle, and CSC senescence.

DNA damage and the expression of proteins blocking the cell cycle increase in stem cells with age, reducing the pool of functionally competent cells [44]. This notion seems to contradict the definition of stem cells. According to traditional views, stem cells possess an unlimited self-renewal capacity, which exceeds the lifespan of the organ and organism. However, this is not the case in the bone marrow [44] and in the heart [3]. CSC aging mediated by telomeric shortening triggers the expression of several gene products, which interfere with the cell cycle but mostly with the G1–S transition. The typical marker of cellular senescence $p16^{INK4a}$ is identified in old CSCs. DNA damage produced by telomere erosion up-regulates p53, which together with $p16^{INK4a}$ represents the molecular phenotype of aged CSCs. Primitive cells positive for $p16^{INK4a}$ have been detected in the heart of patients with cardiac decompensation and signs of precocious organ aging (Figure 17.3). Apoptosis of c-kit-positive undifferentiated cells is very high in these aged hearts and is consistently associated with the expression of $p16^{INK4a}$ [3, 12, 40]. As in the bone marrow [45], the fraction of cycling putative CSCs is increased, suggesting that activated primitive cells undergo a limited number of doubling and then reach cellular senescence and growth arrest.

Telomere function is modulated by specific telomere proteins that participate in the prevention of end-to-end fusion of chromosomes [46]. The two telomeric related factors TRF1 and TRF2 bind preferentially to the DNA sequence located at the junction between the telomeric repeat sequence and the G-strand overhang. Alterations in the binding of TRF1 and TRF2 to DNA occur during cellular senescence [47], and deletion of TRF proteins induces premature senescence in murine and human cells by activating the p53 and $p16^{INK4a}$ pathways, which lead to growth arrest and cell death. Accumulation of G-rich single-stranded DNA fragments, together with attenuation in the expression of TRF1 and TRF2, leads to erosion of telomeres and chromosomal instability [48], possibly promoting the formation of intercellular anaphase bridges. Decreased expression of several telomere-associated proteins has been found in the CSCs of senescent animals and in the human decompensated heart [4, 12].

The average telomeric length provides information on the replicative history of the cell. However, this value cannot be interpreted in terms of the growth reserve of the cell and its ability to divide further in the future. The shortest telomere, and not the average telomeric length, is critical for cell viability and chromosome integrity [49]. Similarly, the fate of a CSC may be linked to the shortest telomere present in the cell. Loss of DNA sequences and telomeric shortening do not affect all telomeres homogeneously. Shortening preferentially occurs in a fraction of telomeres [50]. CSCs may have comparable mean telomeric lengths but exhibit significant differences in the length of individual telomeres.

Conclusions

The adult heart is largely composed of terminally differentiated myocytes. Damaged and old units of this highly specialized compartment of contracting cells are constantly replaced by new, younger elements. The heart is, in fact, a dynamic organ where myocyte death and growth are tightly regulated to maintain physiologic homeostasis. Mitosis and cytokinesis have been recognized in mature myocytes and in poorly differentiated cells with a thin subsarcolemmal halo of myofibrils, in combination with telomerase activity. These *in vivo* findings, together with observations *in vitro*, indicate that replicating myocytes are transient amplifying cells derived from lineage commitment of CSCs. Thus, the heart is a

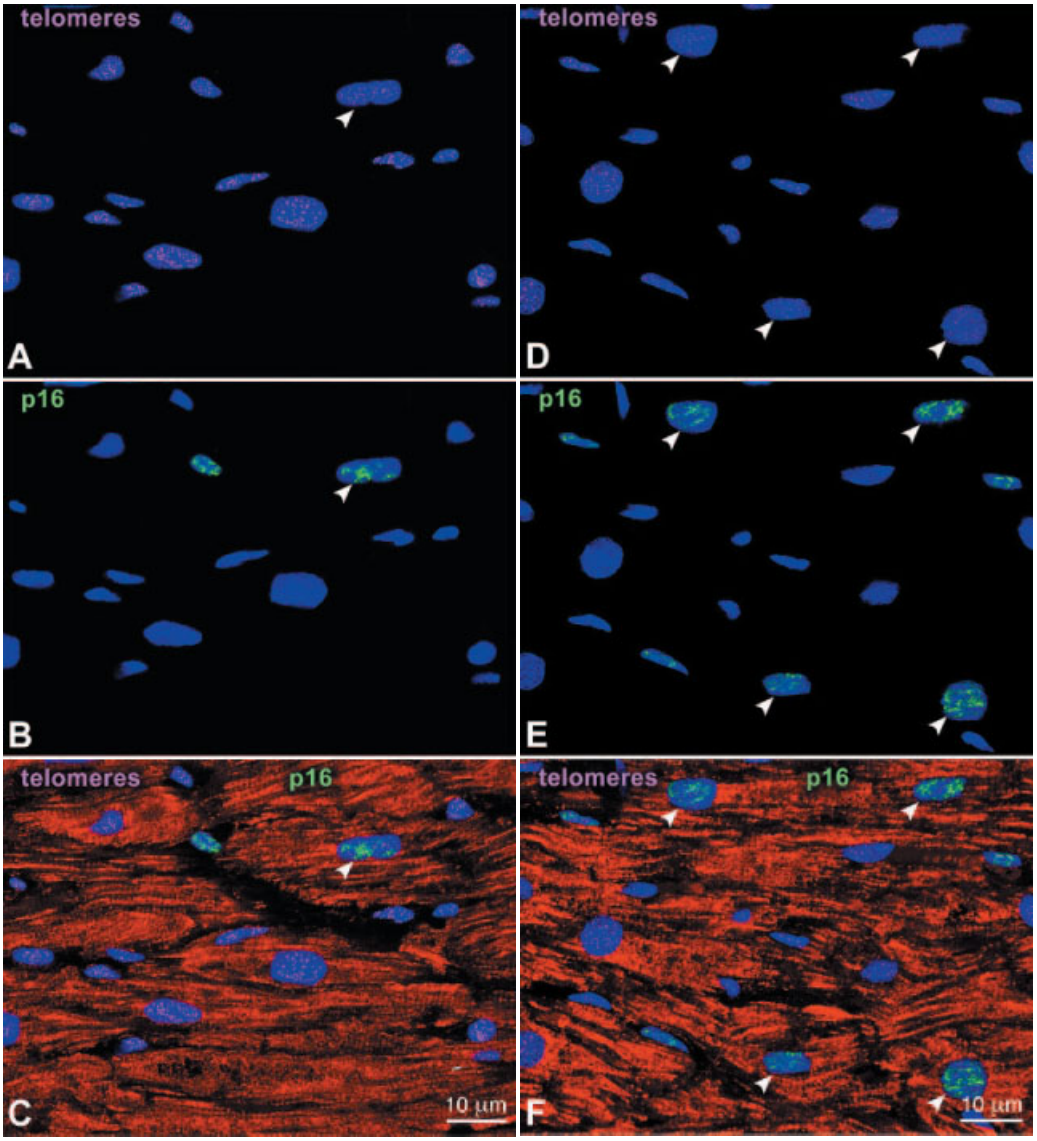


Figure 17.3 Telomere length and p16^{INK4a} activation in normal (A–C) and aged (D–F) failing human hearts. Telomere labeling (A and D; magenta dots) and localization of p16^{INK4a} (B and E; green) in myocyte (C and F; red, α-sarcomeric actin) nuclei. (Adapted from [3].)

self-renewing organ in which the ability to replenish cells is maintained by the existence of a stem cell compartment, which is responsible, when abnormal, for myocardial aging and heart failure.

Acknowledgments

This work was supported, in part, by NIH grants.

References

1 Wei JY. Age and the cardiovascular system. N Engl J Med 1992; 237:1735–1739.
2 Fong LG, Frost D, Meta M, et al. A protein farnesyltransferase inhibitor ameliorates disease in a mouse model of progeria. Science 2006; 311:1621–1623.
3 Chimenti C, Kajstura J, Torella D, et al. Senescence and death of primitive cells and myocytes lead to premature

cardiac aging and heart failure. Circ Res 2003; 93:604–613.
4 Torella D, Rota M, Nurzynska D, et al. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 2004; 94:514–524.
5 Rakusan K. Cardiac growth, maturation, and aging. In Rakusan K (ed.): Growth of the Heart in Health and Disease. New York: Raven Press, 131–164, 1984.
6 Olivetti G, Giordano G, Corradi D, et al. Gender differences and aging: effects on the human heart. J Am Coll Cardiol 1995; 26:1068–1079.
7 Kang P, Izumo S. Apoptosis in heart: basic mechanisms and implications in cardiovascular diseases. Trends Mol Med 2003; 9:177–182.
8 Anversa P, Palackal T, Sonnenblick EH, et al. Myocyte cell loss and myocyte cellular hyperplasia in the hypertrophied aging rat heart. Circ Res 1990; 67:871–885.
9 Takahashi T, Schunkert H, Isoyama S, et al. Age-related differences in the expression of proto-oncogene and contractile protein genes in response to pressure overload in the rat myocardium. J Clin Invest 1992; 89:939–946.
10 Anversa P, Fitzpatrick D, Argani S, Capasso JM. Myocyte mitotic division in the aging mammalian rat heart. Circ Res 1991; 69:1159–1164.
11 Maggioni AP, Maseri A, Fresco C, et al. Age-related increase in mortality among patients with first myocardial infarctions treated with thrombolysis. The investigators of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI-2). N Engl J Med 1993; 329:1442–1448.
12 Urbanek K, Torella D, Sheikh F, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 2005; 102:8692–8697.
13 Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 2006; 113:1451–1463.
14 Dawn B, Stein AB, Urbanek K, et al. Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. Proc Natl Acad Sci USA 2005; 102:3766–3771.
15 Rajindar SS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science 1996; 273:59–63.
16 Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 1999; 18:7719–7730.
17 Higuchi M, Honda T, Proske RJ, Yeh ETH. Regulation of reactive oxygen species-induced apoptosis and necrosis by caspase 3-like proteases. Oncogene 1998; 17:2753–2760.
18 Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979; 59:527–605.
19 Arstall MA, Sawyer DB, Fukazawa R, Kelly RA. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ Res 1999; 85:829–840.
20 Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 1999;15:562–571.
21 Ing DJ, Zang J, Dzau VJ, et al. Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ Res 1999; 84:21–33.
22 Cesselli D, Jakoniuk I, Barlucchi L, et al. Oxidative stress-mediated cardiac cell death is a major determinant of ventricular dysfunction and failure in dog dilated cardiomyopathy. Circ Res 2001; 89:279–286.
23 Frustaci A, Kajstura J, Chimenti C, et al. Myocardial cell death in human diabetes. Circ Res 2000; 87:1123–1132.
24 Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress and aging. Free Radic Biol Med 2000; 29:222–230.
25 Greenberg MM. *In vitro* and *in vivo* effects of oxidative damage to deoxyguanosine. Biochem Soc Trans 2004; 32:46–50.
26 DiMauro S, Tanji K, Bonilla E, et al. Mitochondrial abnormalities in muscle and other aging cells: classification, causes, and effects. Muscle Nerve 2002; 26:597–607.
27 Okorokov AL, Ponchel F, Milner J. Induced N- and C-terminal cleavage of p53: a core fragment of p53, generated by interaction with damaged DNA, promotes cleavage of the N-terminus of full-length p53, whereas ss-DNA induces C-terminal cleavage of p53. EMBO J 1997; 16:6008–6017.
28 Leri A, Liu Y, Wang X, et al. Overexpression of IGF-1 attenuates the myocyte renin-angiotensin system in transgenic mice. Circ Res 1999; 84:752–762.
29 Krajewski S, Krajewska M, Ellerby LM, et al. Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc Natl Acad Sci USA 1999; 96:5752–5757.
30 Li P-F, Dietz R, von Harsdorf R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J 1999; 18:6027–6036.
31 von Harsdorf R, Li P, Dietz R. Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis. Circulation 1999; 99:2934–2941.
32 Hara E, Smith R, Parry D, et al. Regulation of $p16^{CDKN2}$ expression and implication for cell immortalization and senescence. Mol Cell Biol 1996; 16:859–867.

33 Sherr CJ. Cell cycle control and cancer. Harvey Lect 2000–2001; 96:73–92.
34 Hayflick L. The cell biology of aging. J Invest Dermatol 1979; 73:8–14.
35 Sherr CJ, De Pinho RA. Cellular senescence: mitotic clock or culture shock. Cell 2000; 102:407–410.
36 Rubin H. Cell aging *in vivo* and *in vitro*. Mech Ageing Dev1997; 98:1–35.
37 Cristofalo VJ, Allan RG, Pignolo RJ, et al. Relationship between donor age and replicative lifespan in culture, a reevaluation. Proc Natl Acad Sci USA 1998; 95:10614–10619.
38 Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci USA 1995; 92:9363–9367.
39 Greider CW. Cellular responses to telomere shortening: cellular senescence as a tumor suppressor mechanism. Harvey Lect 2000–2001; 96:33–50.
40 Urbanek K, Quaini F, Tasca G, et al. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci USA 2003; 100:10440–10445.
41 Kajstura J, Pertoldi B, Leri A, et al. Telomere shortening is an *in vivo* marker of myocyte replication and aging. Am J Pathol 2000; 156:813–819.
42 Allsopp RC, Weissman IL. Replicative senescence of hematopoietic stem cells during serial transplantation: does telomere shortening play a role? Oncogene 2002; 21:3270–3273.
43 Lansdorp PM. Telomere length and proliferation potential of hematopoietic stem cells. J Cell Sci 1995; 108: 1–6.
44 Geiger H, Van Zant G. The aging of lympho-hematopoietic stem cells. Nat Immunol 2002; 3:329–333.
45 Morrison SJ, Wandycz AM, Akashi K, et al. The aging of hematopoietic stem cells. Nat Med 1996; 2:1011–1016.
46 Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. Science 2002; 295:2446–2449.
47 Hubbard K, Dhanaraj SN, Sethi KA, et al. Alteration of DNA and RNA binding activity of human telomere binding proteins occurs during cellular senescence. Exp Cell Res 1995; 218:241–247.
48 Blasco MA, Lee HW, Hande MP, et al. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997; 91:25–34.
49 Hemann MT, Strong MA, Hao L-Y, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 2001; 107:67–77.
50 Lansdorp PM, Verwoerd NP, van de Rijke FM, et al. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet 1996; 5:685–691.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

18 CHAPTER 18

Bioengineered scaffolds: myocytes, endothelial cells and cardiac repair

Vincent F.M. Segers, MD, *& Richard T. Lee,* MD

Introduction

A substantial effort in cardiac regeneration research has focused on identifying suitable cell types for replacing lost cardiomyocytes and the proliferation and differentiation capabilities of those cells. The rationale behind this research is that the lack of spontaneous cardiac regeneration after myocardial infarction is due to cardiomyocyte terminal differentiation and inability to proliferate, coupled with inadequate availability of progenitor cells capable of forming new cardiomyocytes. Supplementing the damaged heart with progenitor cells or another source of cardiomyocytes is a logical option following this rationale.

However, the vast majority of cells implanted in the myocardium do not survive [1], and those that do survive do not fully integrate into the myocardium as functional cardiomyocytes or endothelial cells, failing to couple electrically or mechanically with remaining myocardium [2]. Some cell types are likely to integrate better than others; however, whichever cell type is selected for implantation into infarcted or injured myocardium will encounter a relatively hostile local environment. As such, one plausible explanation for the failure of mammalian cardiac regeneration could be that although progenitor cells are available in sufficient numbers in the heart, regeneration fails because of a cell-unfriendly microenvironment after myocardial infarction. Thus, to achieve the full potential of cardiac regeneration, we will likely need methods that control the environment into which stem cells are delivered.

Healthy tissue can be considered comprising three dynamic components: (i) cells, (ii) the extracellular matrix (natural scaffold), and (iii) factors such as growth factors that signal to cells. Successful cardiac regeneration therapy will require consideration of all three components and may not be enabled by injection of one cell type alone. Newly formed cells require an extracellular environment providing growth factors, nutrients, mechanical support, and physiological integration in order to contribute to pump function of the heart. Controlling the microenvironment of transplanted cells is challenging, but might be accomplished by bioengineered scaffolds made from modern biomaterials. A traditional definition of biomaterials is that a biomaterial is a nonliving substance used in a medical device, like a joint prosthesis. But the technology of biomaterials has evolved, and the modern definition now includes bioactive substances that are designed to control the biological behavior of cells and tissues. More than simply being compatible with the host and serving a structural role, biomaterials can now instruct cells through microenvironmental cues. Thus, one way to define tissue engineering is that "tissue engineering is the persuasion of the body to heal itself through the delivery, to the appropriate site, of cells, biomolecules and/or supporting structures" [3]. In this chapter, we will consider endothelial cells and fibroblasts as supporting cell types for cardiac tissue engineering. We will summarize the recent progress toward cardiac repair with new injectable biomaterials. Key properties for scaffold selection will be discussed, including developments

in *smart* materials capable of responding to the environment.

Importance of endothelial cells and fibroblasts for preservation of implanted cells

The structural complexity of the myocardium goes far beyond the mere viability of cardiomyocytes. Other cell types like *endothelial cells* have important roles in both the embryonic and adult heart. In the normal mammalian myocardium, every cardiomyocyte is surrounded by a network of capillaries and cardiomyocytes interact directly with adjacent endothelial cells [4]. Cardiomyocytes depend on endothelial cells not only for blood supply but also for paracrine signals and growth factors that control contractility and promote cardiomyocyte organization and survival.

The most obvious role of endothelial cells in cardiac regeneration and tissue engineering in general is oxygen and nutrient supply by *vascularization* of the newly formed tissue (Figure 18.1A). Cell constructs that are developed *ex vivo* usually lack the vascular network that exists in normal tissues, and implanted cardiac tissue will likely be ischemic, at least initially. Therefore, the ability to support vascularization is one of the most important requirements for tissue engineering scaffolds. This concept also applies to injectable scaffolds: cell survival within these scaffolds will depend on recruitment of endothelial cells and new functional vessels. Some materials support vascularization more readily than others. Self-assembling peptides are short peptides that can form a solid scaffold within the myocardium after injection. In many materials including collagen gels and alginate gels, cultured endothelial cells undergo apoptosis, while endothelial apoptosis in self-assembling peptides is less prominent [5]. We showed that after injection of self-assembling peptides in the heart, endothelial cells are recruited and form functional vessels within 4 weeks; in the same time period, few endothelial cells are recruited to the basement membrane material called matrigel [6]. To support angiogenesis in materials not easily vascularized, scaffolds can potentially be engineered with angiogenic growth factors. For instance, porous alginate scaffolds containing microspheres loaded with vascular endothelial growth factor (VEGF) [7] or basic fibroblast growth factor (FGF-2) [8] show increased vascular density compared to scaffolds without growth factors when implanted in hepatic lobes or rat peritoneum, respectively.

The endocardial endothelium plays an important role in cardiac development [4], but endothelial cells also regulate myocardial performance in the adult [4] (Figure 18.1B) through *paracrine roles* of cardiac endothelial cells. Endothelial cells can increase inotropy by converting angiotensin I to angiotensin II and also by secreting endothelin-1 and nitric oxide [4]. These physiological observations are relevant for cardiac regeneration and tissue engineering, as they suggest that vascularization is essential for physiological performance. In fact, engineered heart tissue (EHT) develops a twofold higher contractile force if generated from an unpurified cell preparation than that generated from a pure cardiomyocyte fraction [9]. This suggests that the noncardiomyocyte fraction of cells must be considered in strategies for cardiac regeneration.

Cardiac endothelial cells may also provide important cardiomyocyte survival signals in adult myocardium, since coculture with endothelial cells promotes cardiac myocyte survival [10] (Figure 18.1B). Endocardial and microvascular endothelial cells produce growth factors such as platelet-derived growth factors (PDGFs) and neuregulin that can prevent cardiomyocyte apoptosis [11, 12]. We recently showed that PDGF-BB is an important signaling factor in endothelial–cardiomyocyte cross talk and that the incorporation of PDGF-BB in an injectable scaffold decreased apoptosis and increased cardiac function after myocardial infarction [11]. Endothelial cells also play a poorly understood role in recruiting and organizing cardiomyocytes (Figure 18.1C), similar to the recruiting role of endothelial cells on mural cells during vasculogenesis. Endothelial cells form typical vascular networks with capillary-like tubes when cultured in a three-dimensional scaffold, whereas cardiomyocytes cultured alone form small islands of dying cells. However, when both cell types are cultured together, the endothelial cells form tubular structures and the cardiomyocytes attach to the outside of these vascular tubes [10]. These data suggest that endothelial cells promote the assembly of cardiomyocytes on the capillary endothelial tube either by direct physical interaction or by paracrine

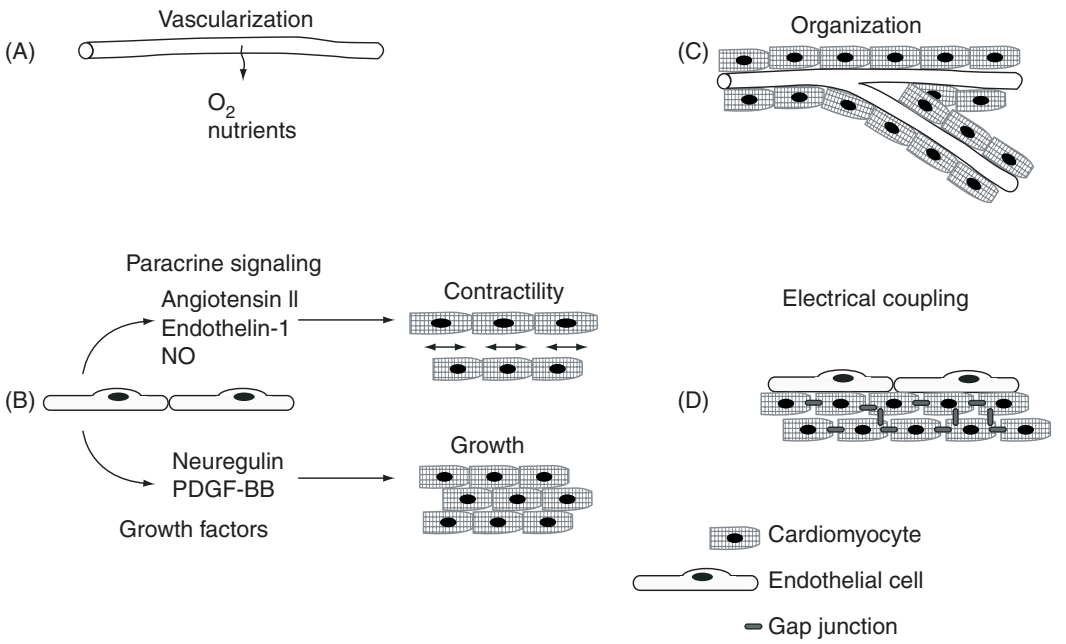


Figure 18.1 Role of endothelial cells in cardiac regeneration. (A) Endothelial cells play a role in cardiac regeneration as building blocks of the vasculature, (B) regulate contractility of cardiomyocytes by secretion of paracrine factors and influence growth of cardiomyocytes during embryogenesis, (C) recruit cardiomyocytes and guide their three-dimensional arrangement in newly formed myocardium, and (D) promote formation of gap junctions between cardiomyocytes. No = nitric oxide; PDGF = platelet-derived growthfactor.

factors. Furthermore, endothelial cells induce formation of gap junctions between cardiomyocytes [10] (Figure 18.1D). In summary, because of their paracrine role for growth and contractility and their role in organization of cardiomyocytes, angiogenesis by endothelial cells may be essential for successful cardiac tissue regeneration.

Cardiac fibroblasts are another important cell population in the heart, both quantitatively and qualitatively, but are often regarded as detrimental since excessive fibrosis is clearly pathological. It is well known that fibroblasts contribute to passive mechanical properties by production and organization of the collageneous matrix. Just as important, fibroblasts provide the anisotropic network of matrix that aligns cardiac myocytes in the orientations crucial for the twisting motion of efficient ventricular function. However, in addition to these structural roles, fibroblasts may have functional roles in the myocardium. Fibroblasts can form gap junctions with cardiomyocytes [13], suggesting that they may play an active role in cardiac electrophysiology. Although fibroblasts do not regulate cardiomyocytes in the same manner as endothelial cells, they might be essential in cardiac tissue engineering and regeneration because of structural contributions and their essential roles in maintaining the dynamic extracellular matrix.

In vitro creation of cardiac tissue

The easiest way to control the microenvironment of implanted cells is developing constructs *in vitro* consisting of cells embedded in three-dimensional scaffolds. These three-dimensional blocks of tissue can then be used for surgical implantation to augment myocardial function, for physiological studies or for *in vitro* drug testing. Constructs created in this manner for cardiac regeneration are often termed EHTs [9]. The biomaterial scaffold that serves as the mechanical and biological support for cell growth and differentiation is a crucial factor in constructing EHT.

Many attempts at engineering myocardium have used *collagen or matrigel* as the scaffold and *fetal or neonatal cardiomyocytes* as a cell source. EHTs can be constructed in different shapes and sizes

and they display functional and morphological properties of differentiated cardiac muscle *in vitro*, although variability in performance can be substantial. Spontaneous contractions start a few days after construction, and applying cyclical stretch in order to mechanically condition the EHT can improve morphological, functional, and mechanical properties [14]. EHT contractions are load dependent [9] and respond to pharmacological stimulation [14]. Most frequently, EHTs are not colonized by a pure population of cardiomyocytes, but mixed cardiac cell populations are used. As noted above, endothelial cells improve survival of cardiomyocytes within and contractility of EHT [9]. *Ex vivo* growth of EHT allows easy addition of growth factors, although the size of EHTs can be limited by oxygen transport. A substantial barrier to the use of EHT for clinical therapy is incorporation of these constructs *in vivo*. Implantation studies in rats indicate that EHT can survive and integrate with the host myocardium [9], but immunosuppressive therapy may be required even after syngeneic transplantation. EHT survival will depend on adequate vascularization and immunological acceptance, and electrical coupling will be required for synchronicity with native cardiac tissue and electrophysiological stability. Finally, mechanical and functional integration should lead to improvement in systolic and diastolic function, since we do not want to sacrifice improvement in one phase of the cardiac cycle for worsening of the other. For these reasons, EHTs may be most useful not for surgical augmentation in patients, but for drug discovery and *in vitro* studies.

Injectable biomaterials

In contrast with *ex vivo* tissue engineering, cell therapy generally does not allow control of the local cellular environment. However, injectable scaffolds can combine advances in materials sciences and progenitor cell biology to modify the cellular microenvironment *in vivo*. Ideally, an injectable myocardial scaffold promotes repair after infarction by providing a matrix support in which neoangiogenesis takes place readily. A number of materials meet this requirement. Christman et al. [15] used a *fibrin* scaffold – known to be an angiogenic environment – as an injectable scaffold after myocardial infarction in rats. Their echocardiography studies showed that "fibrin glue" preserved infarct wall thickness and cardiac function. Furthermore, fibrin glue improved myoblast graft retention and survival, reduced infarct expansion, and induced neovascularization in the infarcted myocardium [15]. These findings were supported by Ryu et al. [16] who showed that implantation of bone marrow mononuclear cells using injectable fibrin matrix enhanced neovascularization in infarcted myocardium compared to cell implantation without matrix. More recently, Kofidis et al. [17] have shown that injection of a mixture of mouse embryonic stem cells and liquid matrigel into the infarcted myocardium restored injured myocardium. Although fibrin and matrigel are injectable, support neovascularization and may improve cardiac function, the fact that they are derived from natural materials renders them less suitable for designing specific biological solutions.

In contrast, synthetic biomaterials can be designed with a variety of biological features. Some short peptides have the unique property of self-organization and self-assembly [18]. Once injected, these materials respond to the physiological environment within the tissue and self-associate in particular patterns to form organized structures. Ionic strength, pH, and physiological salt solutions are such cues (Figure 18.2A) that can guide bioengineered peptides into a mature scaffold. These self-assembling peptides, because of their regular repeats of alternating hydrophilic and hydrophobic amino acids, form stable structures based on protein β-sheets. Following exposure to physiological salt solution, the peptides can spontaneously assemble into a stable macroscopic porous matrix of orderly interwoven nanofilaments [18]. Scaffold formation occurs within minutes after exposure to physiological salt conditions, a property that greatly facilitates *in vivo* microenvironment formation after injection. Cardiac myocytes cultured in these scaffolds exhibit a high degree of spatial organization and differentiation similar to myocardium, with spontaneous and coordinated contractions [10]. Upon injection *in vivo*, these nanofiber microenvironments are spontaneously vascularized [6]. These newly engineered injectable scaffolds hold promise for modifying the microenvironment for suitable progenitor cells with growth factors.

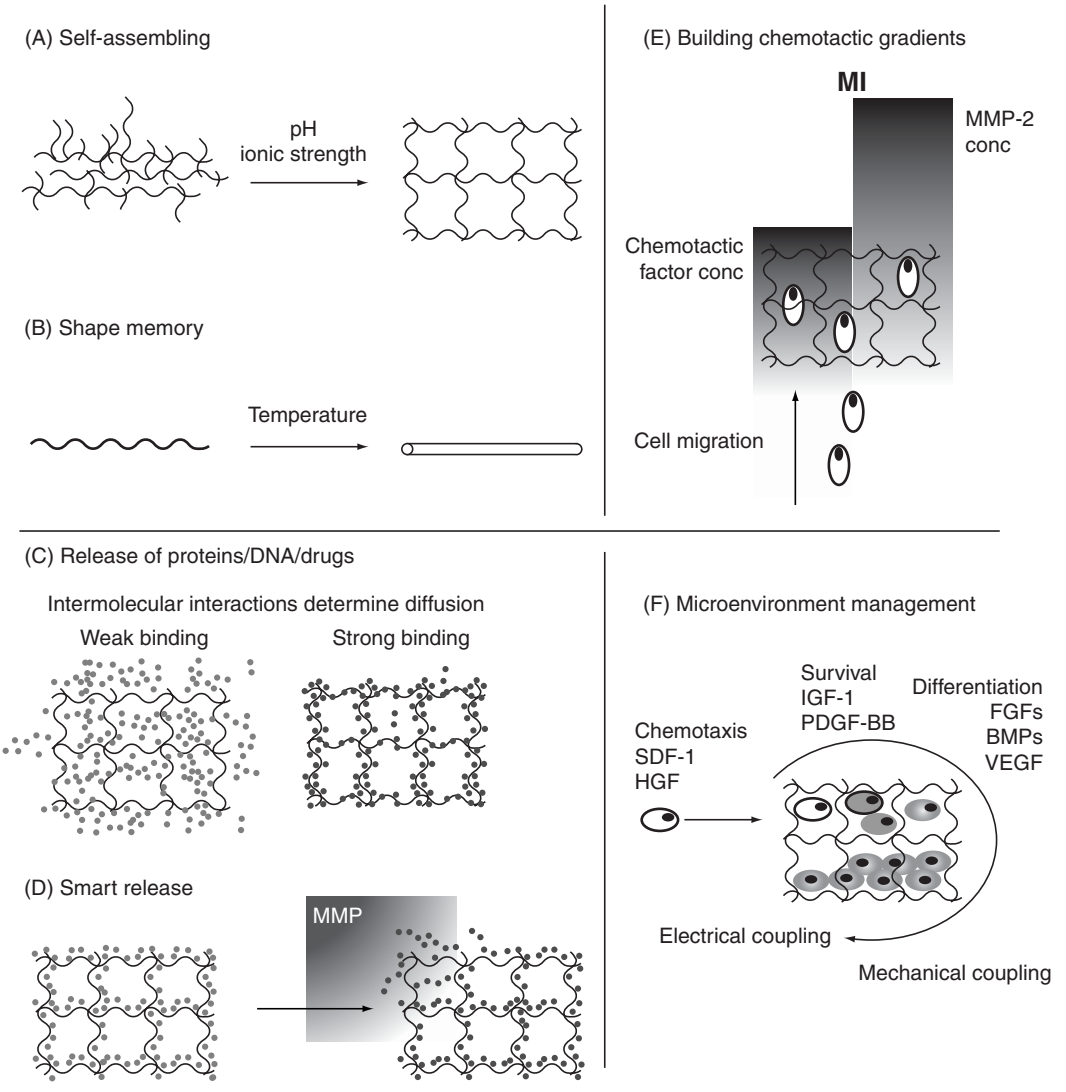


Figure 18.2 Biomaterials for managing the microenvironment. (A) Self-assembling peptides form stable hydrogels. Upon injection to a physiological environment, they form a scaffold of interwoven nanofibers because of the changes in ionic strength and pH. (B) Materials with shape memory transform to a preset form at given temperature. (C) Bioactive factors can be incorporated by noncovalent binding to the scaffold. Depending on the type of reactions between the delivered factor and the scaffold, binding can be weak or strong with, respectively, fast and slow release. (D) Factors can be linked to the scaffold by a substrate peptide sequence for matrix metalloproteinases (MMPs) leading to cleavage and release when MMPs are activated (e.g., after myocardial infarction [MI]). (E) Smart release of a chemotactic factor coupled to the scaffold by a substrate sequence for MMP-2. The concentration of the chemotactic factor will be higher in the neighborhood of infarcted myocardium, where MMP-2 concentrations are elevated. In this way, a gradient of chemotactic factor can be formed to attract progenitor cells toward the infarct zone. (F) Management of the microenvironment by tissue engineering consists of attracting progenitor cells, enhancing their survival, driving their differentiation, and promoting mechanical and electrical coupling. Conc = concentration; SDF = stromal cell-derived factor; HGF = hepatocyte growth factor; IGF = insulin-like growth factor; PDGF = platelet-derived growth factor; FGF = fibroblast growth factor; BMP = bone morphogeneic protein; VEGF = vascular endothelcal growth factor.

Key factors in material selection

The biomaterial scaffold should be able to provide not only a physical support for cells but also the chemical and biological cues needed for functional tissues. For cardiac tissue engineering, the optimal material for the scaffold will guide proliferation, differentiation, organization, and electromechanical coupling of cells. The biomaterial must interact on a molecular level with the cells in a precise and controlled manner, similar to the interactions between cells and the native matrix. At the same time, the basic requirements for a biomaterial should be kept; that is, the materials and their degradation products must be nontoxic and nonimmunogenic. The list of requirements for the ideal material is extensive (Table 18.1), explaining why the optimal scaffold for cardiac regeneration has yet to be designed.

Naturally occurring materials include polypeptides, polysaccharides or composites. The advantage of using naturally occurring building blocks is working with materials of which physiological activities are known, such as selective cell adhesion, mechanical properties or biodegradability. Disadvantages include potential transfer of infectious agents from animals, antigenicity, unstable material supply, and deterioration in the long term. In addition, specific properties of naturally derived materials are difficult to design. Several groups have studied scaffolds composed of natural polymers such as collagen [9], the major constituent of the cardiac extracellular matrix. Reasonable mechanical results were obtained with collagen-based grafts or "patches" containing beating cardiomyocytes (EHT). Alginate is a negatively charged polysaccharide from seaweed which forms hydrogels in the presence of calcium and has a high porosity enabling high seeding densities, so alginate-based scaffolds may be used [19].

Table 18.1 Optimal features of materials for cardiac tissue engineering.

Innate properties
Noncytotoxic
Nonimmunogenic
Noninfectious
Biodegradable with nontoxic products
Porosity
Mechanical (strength/elasticity)
Injectable
Biological properties
Support cell attachment
Support cell survival and proliferation
Drive migration of progenitors
Support development of functional vasculature
Support mechanical and electrical integration
Smart properties
Shape memory
Trigger release of growth factors

Drawbacks of natural materials isolated by extraction (e.g., collagen) can be overcome by selecting fragments with specific functions instead of using crude materials. Recently, Ito et al. [20] mapped the D4 domain of human collagen II as most critical for supporting migration of chondrocytes and used this information to engineer a collagen-like protein consisting of tandem repeats of the D4 domain (mD4 collagen). Cartilaginous constructs of chondrocytes cultured in polyglycolic acid (PGA) scaffolds coated with mD4 collagen proved to be superior to constructs of chondrocytes cultured in bare PGA scaffolds or scaffolds coated with natural collagen [20]. Hence, although biological materials can be difficult and expensive to engineer and purify, known properties of natural materials can be used as a template for synthetic materials to circumvent drawbacks associated with natural scaffolds and to select and amplify the desired properties.

Synthetic materials can be manufactured on a large scale, and their structure and mechanical properties can be more easily controlled and manipulated than natural materials. A potential drawback of most synthetic materials is their lack of specific cell-recognition signals. Cells adhere to and interact with their extracellular environment via cell surface receptors including *integrins*, and their ability to activate associated downstream signaling pathways depends on adhesion complexes formed between cells and matrix. To improve cellular adhesion, cell-adhesion motifs may be engineered into biomaterials [21]. These sites have to be present in sufficient number and at the same time have to be properly spaced in order to organize cardiomyocytes and endothelial cells. Recognition motifs are formed by specific amino acid sequences of which the Arginine–Glycine–Asparagine (RGD) sequence, commonly found in fibronectin, laminin, and other matrix proteins, is

best characterized. Polylactic acid, PGA, and their copolymer polylactic-co-glycolic acid lack natural cell recognition motifs. Cannizzaro et al. [21] coupled the RGD–peptide–biotin sequence to polymers by a biotin–streptavidin–biotin linkage, resulting in increased spreading of endothelial cells in these polymers. Organization of RGD on the matrix is as important as the total number of available RGD sites: it has been shown that cell spreading and proliferation of osteoblasts in alginate gel are altered by the spacing between RGD moieties [22].

The surface properties of a biomaterial not only affect cell spreading and proliferation but also differentiation. Differentiation of mesenchymal stem cells is dependent on specific coating of the biomaterial [23]. Mesenchymal stem cells are sensitive to surface chemistry, and N-enriched surfaces by means of glow discharge plasma techniques can influence human mesenchymal stem cell responses, ultimately affecting differentiation toward osteoblasts.

An important element in the development of every bioactive material is control of porosity. This property has not been studied extensively in cardiac tissue engineering, but conclusions can be drawn from osteogenesis research [24] and musculoskeletal tissue engineering [25]. *In vitro*, small pore size scaffolds (40 μm) contain more cells and stimulate osteogenesis by suppressing cell proliferation and forcing cell aggregation [24]. However, pores smaller than 100 nm result in decreased diffusion of oxygen, nutrients, and growth factors, leading to poor survival of implanted cells [25]. Larger pores (100 μm) are beneficial for cell migration and proliferation in the scaffold [24, 25], but pores larger than the size of one endothelial cell are difficult to bridge and lead to decreased angiogenesis; large pore sizes may also compromise mechanical properties. Thus, a balance in porosity must be reached depending on the repair strategy, as porosity can affect cell migration, angiogenesis, rate of remodeling, and rate of degradation of the scaffold material.

Smart biomaterials

The defining characteristic of smart biomaterials is *responsiveness* to environmental cues. These cues can include pH, ionic strength, temperature, or enzymes that trigger responses such as protein release (Figures 18.2A–2D). Smart materials are promising for accomplishing stem cell differentiation and cellular chemotaxis, which can require sequences of signaling molecule release.

Although biomaterials provide necessary mechanical support and adhesion sites, they usually do not guide cellular phenotype as growth factors do. Incorporation of growth factors will greatly improve the ability of scaffolds to form microenvironments for stem cells and cardiomyocytes. In addition, biological signaling molecules are often unstable *in vivo*; therefore, it may be necessary to administer them together with carrier molecules to enhance their *in vivo* activity [11]. Protein delivery systems must meet criteria that are *specific* to the application (vasculogenesis, differentiation or chemotaxis) and to the protein. Endogenous levels of the protein should be known and increasing the amount above this level must have an additional beneficial effect. Delivery of a protein to receptors that are already optimally activated by the endogenous protein may be detrimental, as has been described for overexpression of VEGF [26].

The delivered factor must be targeted to a specific cell population, with minimal influence on other cell types. The ideal chemotactic factor attracts only one cell type. Stromal-cell-derived factor-1 (SDF-1) is one example of a cardiac repair stem cell chemoattractant [27]. The ability of SDF-1 to attract hematopoietic stem cells to the heart has been extensively described; however, SDF-1 is also a chemotactic factor for T lymphocytes. Moreover, the mere presence of a chemotactic factor in a material or a region of myocardium is insufficient for efficient cell migration. Cells migrate in a certain direction driven by subtle differences in chemokine gradients across the cells through a mechanism called spatial sensing. Gradients of chemokines may be achieved by attachment of chemokines to bioengineered scaffolds, using a linker peptide containing a substrate sequence for metalloproteinases (MMPs). MMPs recognize specific peptide sequences in proteins which can be incorporated into recombinant proteins. MMPs are activated after myocardial infarction, which may lead to release of a linked protein in a spatial and timely controlled manner. Feasibility of this release method has been proved by linking methotrexate to dextran via a substrate sequence for MMP-2 and MMP-9. MMP-2 and MMP-9 are

expressed by certain tumor cells and this delivery method led to spatial and temporal control of release of methotrexate [28]. Release of a chemotactic factor linked to the scaffold via a substrate sequence for MMP-2 will theoretically occur at a higher rate in regions closer to the infarcted region, leading to a well-defined gradient of the factor (Figure 18.2E).

Growth factors can be linked to a biomaterial scaffold by biotin molecules or by covalent attachment to the material. Modifying interactions between proteins to be delivered and the scaffold will influence the timing of release (Figure 18.2C). The kinetics of release of a factor must be considered along with the total binding capacity of the material. We have demonstrated that self-assembling peptides can deliver pharmacological doses of PDGF [11]: binding capacity of the peptides for PDGF was at least 10 times higher than that necessary for physiologic effects. Recently, Behfar and Terzic defined a "cardiogenic cocktail" [29] consisting of ten substances necessary for induction of transdifferentiation of adult mesenchymal stem cells into cardiomyocytes *in vitro*. This cocktail included inductors of embryonic cardiogenesis bone morphogenetic protein 2, transforming growth factor β1, and retinoic acid; growth factors like insulin-like growth factor 1, VEGF, and FGF-4; and other factors like tumor necrosis factor α, leukemia inhibitory factor, interleukin 6, and α-thrombin. As with cardiogenesis in the embryo, formation of new cardiomyocytes in the adult will require delivery of growth factors in a highly orchestrated manner. The study by Behfar and Terzic provides another illustration that one factor alone will not suffice [29]. Delivery of many factors together in a controlled manner is a daunting task requiring extensive knowledge of the biology and chemistry of each factor.

Besides protein delivery, engineered scaffolds have potential for improving or controlling gene transfer. Synthetic carriers, including peptide-based delivery systems, are being developed in several laboratories as promising alternatives to using viruses as vectors. Because of their exceptional adaptability, peptide-based vectors are particularly amenable to this approach. Zhang et al. [18] developed a series of surfactant peptides consisting of a hydrophobic tail attached to a polar group (positively charged residues at the C or N terminus). These peptides self-assemble in water to produce nanovesicles and nanotubes [30]. When placed in a DNA solution, the positively charged peptides self-assembled into a tube, encapsulating the negatively charged DNA. In this manner, DNA could be delivered to cells and the surface of DNA can be tagged with a marker that is specific to a particular cell type [30].

Conclusions

Biomaterials can be engineered in different ways to create a cardiogenic microenvironment and promote successful *integration* of cells. The first requirement is successful vascularization, not only for oxygen and nutrient supply but also for paracrine and organizational actions of endothelial cells. It is clear that cardiac repair is a reasonable goal, but much more knowledge regarding the differentiation of stem cells to cardiac myocytes is necessary for advancement in this field. With this advance in knowledge, we can then provide the biomaterial design to instruct cells in the local microenvironment.

Critical for bioengineered scaffolds is selection of suitable materials supporting cell spreading and survival. Furthermore, factors must be incorporated to attract cells and promote survival, differentiation, and electromechanical integration of cells in the newly formed tissue. For instance, chemotactic signals can be incorporated to attract progenitor cells and growth factors can be included to promote survival and/or transdifferentiation. These bioactive factors have to be delivered in a highly orchestrated manner leading to true *management of the microenvironment* (Figure 18.2F). Another favorable feature of biomaterials is injectability to avoid the need for surgical implantation. Eventually, successful cardiac regeneration will depend not only on the cell strategy that we use, but also on the level of control and integration that can be obtained by managing the microenvironment.

References

1 Itescu S, Schuster MD, Kocher AA. New directions in strategies using cell therapy for heart disease. J Mol Med 2003; 81:288–296.

2 Dowell JD, Rubart M, Pasumarthi KB, et al. Myocyte and myogenic stem cell transplantation in the heart. Cardiovasc Res 2003; 58:336–350.

3 Williams DF. To engineer is to create: the link between engineering and regeneration. Trends Biotechnol 2006; 24:4–8.

4 Brutsaert DL. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. Physiol Rev 2003; 83:59–115.

5 Narmoneva DA, Oni O, Sieminski AL, et al. Self-assembling short oligopeptides and the promotion of angiogenesis. Biomaterials 2005; 26:4837–4846.

6 Davis ME, Motion JP, Narmoneva DA, et al. Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells. Circulation 2005; 111:442–450.

7 Kedem A, Perets A, Gamlieli-Bonshtein I, et al. Vascular endothelial growth factor-releasing scaffolds enhance vascularization and engraftment of hepatocytes transplanted on liver lobes. Tissue Eng 2005; 11:715–722.

8 Perets A, Baruch Y, Weisbuch F, et al. Enhancing the vascularization of three-dimensional porous alginate scaffolds by incorporating controlled release basic fibroblast growth factor microspheres. J Biomed Mater Res A 2003; 65:489–497.

9 Eschenhagen T, Zimmermann WH. Engineering myocardial tissue. Circ Res 2005; 97:1220–1231.

10 Narmoneva DA, Vukmirovic R, Davis ME, et al. Endothelial cells promote cardiac myocyte survival and spatial reorganization: implications for cardiac regeneration. Circulation 2004; 110:962–968.

11 Hsieh PC, Davis ME, Gannon J, et al. Controlled delivery of PDGF-BB for myocardial protection using injectable self-assembling peptide nanofibers. J Clin Invest 2006; 116:237–248.

12 Kuramochi Y, Cote GM, Guo X, et al. Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. J Biol Chem 2004; 279:51141–51147.

13 Camelliti P, Borg TK, Kohl P. Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res 2005; 65:40–51.

14 Fink C, Ergun S, Kralisch D, et al. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. FASEB J 2000; 14:669–679.

15 Christman KL, Vardanian AJ, Fang Q, et al. Injectable fibrin scaffold improves cell transplant survival, reduces infarct expansion, and induces neovasculature formation in ischemic myocardium. J Am Coll Cardiol 2004; 44:654–660.

16 Ryu JH, Kim IK, Cho SW, et al. Implantation of bone marrow mononuclear cells using injectable fibrin matrix enhances neovascularization in infarcted myocardium. Biomaterials 2005; 26:319–326.

17 Kofidis T, de Bruin JL, Hoyt G, et al. Injectable bioartificial myocardial tissue for large-scale intramural cell transfer and functional recovery of injured heart muscle. J Thorac Cardiovasc Surg 2004; 128:571–578.

18 Zhang S, Gelain F, Zhao X. Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures. Semin Cancer Biol 2005; 15:413–420.

19 Dar A, Shachar M, Leor J, Cohen S. Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. Biotechnol Bioeng 2002; 80:305–312.

20 Ito H, Steplewski A, Alabyeva T, Fertala A. Testing the utility of rationally engineered recombinant collagen-like proteins for applications in tissue engineering. J Biomed Mater Res A 2006; 76:551–560.

21 Cannizzaro SM, Padera RF, Langer R, et al. A novel biotinylated degradable polymer for cell-interactive applications. Biotechnol Bioeng 1998; 58:529–535.

22 Lee KY, Alsberg E, Hsiong S, et al. Nanoscale adhesion ligand organization regulates osteoblast proliferation and differentiation. Nano Lett 2004; 4:1501–1506.

23 Mwale F, Wang HT, Nelea V, et al. The effect of glow discharge plasma surface modification of polymers on the osteogenic differentiation of committed human mesenchymal stem cells. Biomaterials 2006; 27:2258–2264.

24 Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials 2005; 26:5474–5491.

25 Karande TS, Ong, JL, Agrawal CM. Diffusion in musculoskeletal tissue engineering scaffolds: design issues related to porosity, permeability, architecture, and nutrient mixing. Ann Biomed Eng 2004; 32:1728–1743.

26 Ozawa CR, Banfi A, Glazer NL, et al. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. J Clin Invest 2004; 113:516–527.

27 Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003; 362:697–703.

28 Chau Y, Tan FE, Langer R. Synthesis and characterization of dextran-peptide-methotrexate conjugates for tumor targeting via mediation by matrix metalloproteinase II and matrix metalloproteinase IX. Bioconjug Chem 2004; 15:931–941.

29 Behfar A, Terzic A. Derivation of a cardiopoietic population from human mesenchymal stem cells yields cardiac progeny. Nat Clin Pract Cardiovasc Med 2006; 3(suppl 1):S78–S82.

30 Fairman R, Akerfeldt KS. Peptides as novel smart materials. Curr Opin Struct Biol 2005; 15:453–463.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

CHAPTER 19

Gene therapy and cellular therapy in cardiac repair

Mark A. Sussman, PhD

Introduction

Repairing and restoring myocardial structure and function represents an ideal outcome for the treatment of cardiomyopathy. Current therapeutic interventions rely predominantly upon pharmacologic strategies to slow the progression of heart failure, but the ultimate destination for many of these patients will be an operating room where they will receive either a cardiomyoplasty, a mechanical assist device, or a transplanted donor heart. The cost, invasiveness, shortage of suitable donor organs, and compromised quality of life are just a few of the many issues stemming from the current approaches to treating advanced heart failure. An attractive alternative vision for treating these patients would be the use of repair processes that, rather than prop up and maintain damaged organ function, would enable the replacement and repair of the compromised tissue on a cellular level. Over the past decade, a concerted effort by scientists and clinicians has advanced the use of gene therapy as an approach to treating heart failure, which has been reviewed extensively in recent publications [1–6]. These studies have shown that genetic reprogramming of the myocardium can be used to prevent cell death, inhibit maladaptive remodeling, enhance hemodynamic function, promote angiogenesis, and block deleterious signaling [7–15]. Although gene therapy approaches remain a popular avenue for delineating the role of proteins in cardiac repair and rescue, many practical aspects of gene therapy, such as delivery and targeted expression in the myocardium, regulation of gene expression, and persistence of expression, have hampered implementation of myocardial gene therapy for the treatment of heart failure. However, recent discoveries related to regeneration and repair of the myocardium, using stem cells, have shifted the paradigm of treatment for myocardial disease [16–21]. Discoveries linking stem cell-based therapies to improvements in myocardial performance have invigorated the field, but current limitations in stem cell-based approaches present significant barriers. This chapter will concentrate on existing challenges in stem cell-based treatment and how these may be overcome by incorporation of gene therapy, resulting in a combinatorial approach that uses genetic engineering to potentiate stem cell activity for myocardial repair.

Stem cell-based therapeutics for the treatment of myocardial damage or disease originated with findings from seminal studies on bone marrow-derived cells [22] which subsequently transitioned into studies using stem cells derived from the heart [23, 24] The ultimate origin of cardiac stem cells remains speculative, but there is no dispute that such cells exert powerful recuperative effects upon the myocardium. Published evidence supports direct and indirect mechanisms of repair mediated by adoptively transferred cells, either by direct engraftment into the host tissue or recruitment and facilitation of repair by activation of endogenous cells. Additionally, the transferred cell population supports survival and protection of the existing myocardial tissue at risk which, in turn, diminishes the area of damage and promotes preservation of cell viability and tissue integrity.

Damaged myocardium is a hostile environment for cells attempting to execute reparative and regenerative processes. Under conditions of acute injury

by ischemia or infarction, cells entering into the affected region may be challenged with hypoxia, oxidative stress, cytotoxic factors, inflammatory processes, deranged extracellular signaling stimuli, and abnormal mechanical stress. The type and intensity of stresses in the damaged region also varies over time as the wounded tissue undergoes cellular death, tissue remodeling, and eventual scarring. In comparison, a chronically failing heart potentially suffers from high levels of catecholamine stimulation, increased cell death and turnover, altered metabolism, elevation of calcium-dependent signaling, and desensitization to normal autocrine and paracrine signaling pathways. In the face of such inhospitable conditions, it is not surprising that many studies find that cells adoptively transferred into this region are lost by attrition as these cellular immigrants die or meander to other locations [25–27]. To encourage survival and long-term persistence, two options are available: (1) modify the environment to render it more welcoming or (2) arm the settlers with extraordinary capabilities to withstand adverse conditions. Both of these approaches have been successfully employed (often in combination) to enhance the efficiency of cell transfer and the efficacy of myocardial repair.

Option 1: modify the environment

The challenge of survival for stem cells in damaged myocardium can be ameliorated by genetic engineering approaches designed to manipulate conditions in the afflicted region. Successful gene therapy approaches to inhibit cardiomyopathy operate by blunting processes of cell death and deleterious remodeling. Until recently, the consequences of gene therapy approaches were evaluated primarily in the context of preventing damage, whereas the contribution of endogenous repair mechanisms was overlooked. Thus, gene therapy approaches to blunt oxidative stress by overexpression of proteins such as heme oxygenase-1 [28–30], catalase [31], or superoxide dismutase [32] can all be thought of as ways to decrease free-radical production that can provoke apoptotic death of stem cells. Blunting inflammatory responses and associated destruction of involved tissue mediated through molecules such as tumor necrosis factor α [33], interleukin 8 [34], endothelial nitric oxide synthase [35], or NFκB [36, 37] activation also contribute to a more favorable seeding ground for stem cells. Part and parcel of this approach is the inhibition of cell death by directing survival signaling in the myocardium via expression of genes such as Bcl-2 [38], Akt [39, 40], hepatocyte growth factor (HGF) [41], cardiotrophin [42], or adrenomedullin [43]. Overcoming cell death can also be accomplished by inhibiting proapoptotic agents such as Bad [44, 45], Bax [46], caspases [47, 48], p53 [49], or Fas ligand [50]. Collectively, all these manipulations enhance myocardial survival in the wake of pathological insults, but they also promote a more salubrious environment for the engraftment and persistence of invading stem cells.

The most widely exploited form of myocardial gene therapy involves the use of proangiogenic factors to promote neovascularization of ischemic or infarcted tissue. Paracrine factors including vascular endothelial growth factor (VEGF) [11, 51], fibroblast growth factor (FGF) [52], or HGF [10, 41] have all been widely used in experimental and clinical gene transfer protocols. Although employed because of their angiogenic properties, these factors can also influence cell survival, growth, and trafficking [53] Benefits obtained through the use of these paracrine factors reside with their multifaceted actions, although the relative contributions of these diverse biological effects in the phenotypic outcome remain poorly understood. This paucity of clear mechanistic understanding is the likely reason for the inconclusive results obtained in the numerous clinical trials that used gene therapy to promote therapeutic angiogenesis in patients with myocardial ischemia [54].

Option 2: protect and potentiate the stem cell population

Enhancing the survival, engraftment, and growth of stem cells allows for modification of the stem cell population rather than the local environment of the myocardium. This approach has been employed in three predominant forms: (1) increasing stem cell resistance to cell death, (2) engineering stem cells to produce factors that enhance nurturing from resident tissue, and (3) programming the cells with increased compatibility or commitment to functional activity prior to delivery. *Ex vivo* manipulation of isolated stem cell populations

by gene transfer, somatic cell (nuclear reprogramming), or preconditioning in coculture with feeder layers or soluble factors are typically used to influence the stem cells. These "potentiated" stem cells are subsequently introduced into the recipient heart, which in most cases has been pathologically challenged by ischemic or infarction injury. Gene delivery vectors are often chosen for integration into the host-cell DNA (retroviruses, adeno-associated viruses) that enables persistent expression in daughter cell progeny, rather than adenoviruses or conventional plasmid transfections that are maintained in episomal form and subsequently diluted away by mitotic activity. However, episomal gene constructs can express for several days to a couple of weeks prior to the loss of the virally encoded transgene, which is often more than the sufficient time to allow for engraftment and/or beneficial modification of the myocardial environment. Genes employed for potentiating stem cell survival upon delivery include HGF [55, 56], VEGF [57, 58], FGF [59], heme oxygenase-1 [60], sonic hedghog [61], transforming growth factor β [62], and Akt/PKB [63]. All of these studies have reported enhancement of survival, engraftment, and improvement in cardiac remodeling/hemodynamic function through the use of genetically engineered cells versus their naïve counterparts.

Combined effects that enhance stem cell engraftment

Paracrine factors are important contributors to the beneficial effect of adoptively transferred stem cells [64]. It is important to keep in mind that genetically altered stem cells are likely to be elaborating factors that, in addition to enhancing the survival of the donated population, will also be exerting beneficial effects on the surrounding host tissue in proximity to the injection site. In such cases, the overall effect may be the combined enhancement of stem cell population survival as well as an enhancement of the local tissue environment. Such is the case with cells reprogrammed with constitutive activation of Akt/PKB kinase [65].

Various mix-and-match approaches will likely be increasingly popular as we delineate the respective roles of specific genes and cell populations in the regenerative/reparative process. Examples include combining stem cells with growth factors at delivery [66, 67], driving preimplantation commitment of the stem cells to favor myocardial-associated lineages [68], and the use of trafficking factors to enhance recruitment of local stem cell populations [69].

Engineering a younger heart with nuclear-targeted Akt kinase

In addition to the aforementioned gene therapy approaches for enhancing cell survival or improving local tissue conditions, another possible avenue for increasing stem cell regenerative capacity is expansion of the stem cell population by increased mitotic activity. Previous attempts to modify cardiomyocyte proliferation by genetic engineering have met with mixed results that have failed to produce a clinically relevant treatment approach [70, 71] Our group has been examining the ability of Akt/PKB kinase modified by nuclear targeting to enhance the survival and proliferation of the cardiac stem cell population. These studies grew from seminal observations made in transgenic mice created with cardiac-specific expression of nuclear-targeted Akt (Akt/nuc) and it was found that the mice were resistant to apoptotic challenge and possessed a hypercellular myocardium without a concomitant increase in cardiac mass [72, 73]. The Akt/nuc myocardium exhibits increased frequencies of cells expressing the stem cell marker c-kit as well as cells expressing c-kit in combination with Nkx 2.5 or MEF2C. Expression of transcription factors MEF2C or Nkx 2.5 identifies cells committed to the myocyte lineage. The number of c-kit$^+$ cells in Akt/nuc samples present in regions of the ventricular midwall or atria showed statistically significant increases of 2.1- and 1.7-fold, respectively (Figure 19.1). Similarly, significant increases of 1.9- or 4.1-fold were found for c-kit$^+$/Nkx 2.5$^+$ cells in the ventricular midwall or atria, respectively, for Akt/nuc hearts relative to nontransgenic samples. These values correlate with the demonstration of a significant 2.9-fold increase in c-kit$^+$/MEF2C$^+$ cells in the midwall region of Akt/nuc samples relative to nontransgenic controls.

Comparison of cytokine mRNA transcript expression by quantitative real-time PCR array analyses between Akt/nuc transgenic heart samples

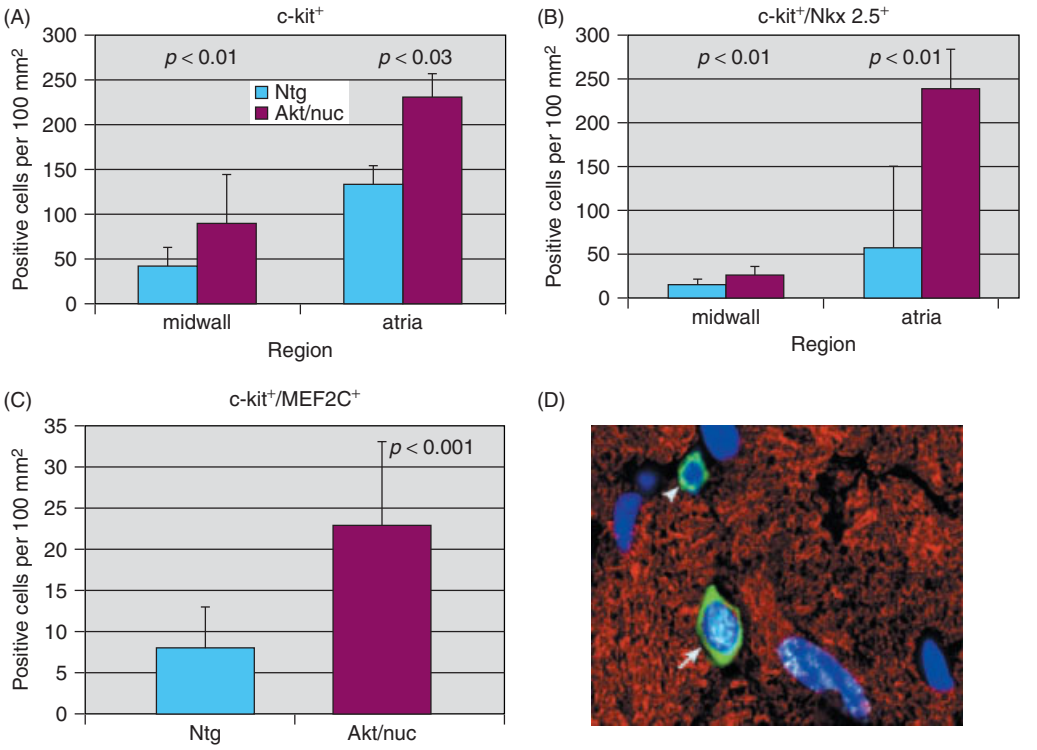


Figure 19.1 Expansion of the stem cell and cardimyocyte progenitor cell populations in Akt/nuc myocardium. Quantitation of cells in the myocardium of Ntg and Akt/nuc hearts immunoreactive with antibodies to (A) c-kit alone, (B) c-kit and Nkx 2.5, or (C) c-kit and MEF2C. Assessment of c-kit$^+$ as well as c-kit$^+$/Nkx 2.5$^+$ populations is subdivided by regional distribution in either ventricular midwall or atria. Calculations are based upon a total area of 500 mm^2 from 10 Ntg or 600 mm^2 from 15 Akt/nuc hearts. Micrograph (D) depicts typical view of myocardium labeled for c-kit$^+$ (green), MEF2C (white), nuclei (blue), and sarcomeric actin (red) with arrowhead indicating a c-kit$^+$ cell and the arrow indicating a c-kit$^+$/MEF2C$^+$ cell. Statistically significant differences between Ntg and Akt/nuc are shown in the graphs. Akt/nuc = nuclear targeted AK+; Ntg = nontransgenic.

versus age- and gender-matched nontransgenic controls (Figure 19.2, solid bars) reveal striking similarities in the transcriptional profile between the Akt/nuc and neonatal heart mRNA changes relative to adult Ntg control samples (Figure 19.2, solid versus dotted bars). Thus, Akt/nuc influences transcriptional reprogramming of cytokine expression in the myocardium that shares similarities with that found in the neonatal heart.

The combination of enhanced resistance to apoptotic cell death and increased proliferative potential makes stem cells modified with cardiac-specific expression of Akt attractive for regenerative cardiac cellular therapy. Pilot experiments show marked engraftment, persistence, and commitment of adoptively transferred stem cells derived from Akt/nuc transgenic mice into infarcted myocardium or recipient mice (Figure 19.3), pointing the way for future studies to examine the application of Akt/nuc genetic engineering in a clinical setting.

Conclusions and future directions

The promise of gene therapy for the treatment of heart failure has yet to be fulfilled. Some of the persistent issues in gene therapy of the myocardium, such as regulation of vector delivery, controlling expression, and long-term persistence, may be resolved by combining gene therapy with stem cell populations that can serve as factories for production of beneficial paracrine factors as well as partners in the process of engraftment and regeneration.

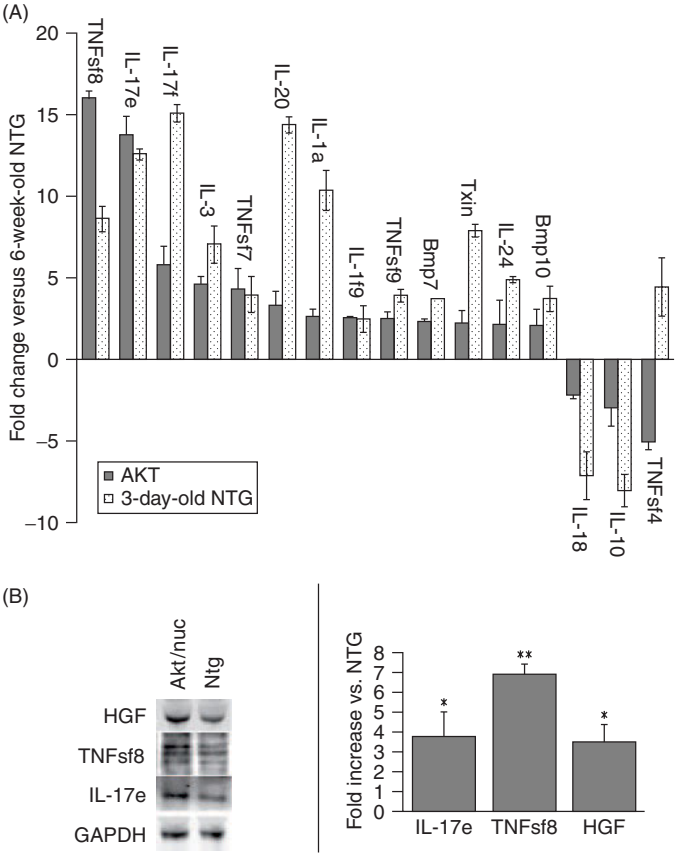


Figure 19.2 Induction of cytokine and growth factor expression in Akt/nuc myocardium. (A) PCR-base microarray analysis of mRNA expression of cytokines and growth factors demonstrating expression changes in myocardial samples from Akt/nuc in comparison to nontransgenic (Ntg) samples. (B) Immunoblot and quantitation of protein expression from Akt/nuc and Ntg myocardial lysates. Significant increases are noted for TNFsf8, IL-17e, and HGF. Results represent averaged values for analyses performed on three separate individual heart samples from either Akt/nuc or age- and gender-matched Ntg controls. *$p < 0.01$; **$p < 0.05$. Txin = Taxillin; Bmp7 = Bone morphogenetic protein 7; GAPDH = Glyceraldehyde-3- phosphate dehydrogenase. Akt/nuc = nuclear targeted Akt; HGF = hepatocyle growth factor; TNF = tumor necrosis factor; IL = interleukin.

Joining the recent observations regarding cardiac progenitor cell biology together with Akt kinase signaling opens novel possibilities for the manipulation of myocardial cell survival, regeneration, and aging. Indeed, our Akt/nuc prompts significant changes in cytokine expression in the hearts of transgenics reminiscent of neonatal transcription profiles for factors known to mediate cell survival, proliferation, and differentiation. There are new frontiers just beginning to be explored, such as genetic reprogramming of stem cells by somatic cell nuclear transfer creation of autologous cell populations for myocardial regeneration [74] or engineering of pacemaker cells to treat arrhythmias [75]. Thus, goals of ongoing studies are to (1) define specific molecular mechanisms responsible for the beneficial effects of genetic engineering for stem cells and myocardial tissues, (2) expand the potential application of genetic modification of cardiac progenitor cells and myocytes for clinically-relevant treatment, and (3) use gene therapy in combination with stem cells to promote recovery from cardiomyopathic injury and to antagonize the progressive deterioration of hemodynamic performance in cardiomyopathies as well as in normal aging.

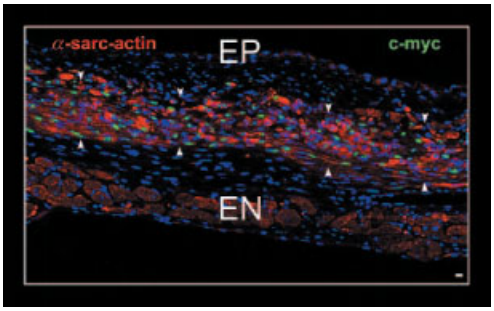


Figure 19.3 Myocardial regeneration by stem cells derived from transgenic mice created with cardiac-specific expression of nuclear-targeted Akt/PKB kinase. Confocal micrograph showing epicardial (EP) and endocardial (EN) surfaces of infarcted myocardium that received intramyocardial injection of stem cells derived from transgenic nuclear-targeted Akt mice. A regenerating band of myocardium is seen (demarcated by arrowheads) wherein the adoptively transferred cells can be identified by immunolabeling for a c-myc tag (green). Many cells in the regenerating band exhibit coincident expression of the myc tag as well as α-sarcomeric actin (red) indicative of commitment to the cardiomyocyte lineage. Nuclei in the section are labeled in blue. Bar in lower right corner of micrograph equals 10 μm.

References

1 Jones JM, Koch WJ. Gene therapy approaches to cardiovascular disease. Methods Mol Med 2005; 112:15–35.

2 Rosen MR, Brink PR, Cohen IS, Robinson RB. Genes, stem cells and biological pacemakers. Cardiovasc Res 2004; 64:12–23.

3 del Monte F, Kizana E, Tabchy A, Hajjar RJ. Targeted gene transfer in heart failure: implications for novel gene identification. Curr Opin Mol Ther 2004; 6:381–394.

4 Hoshijima M. Gene therapy targeted at calcium handling as an approach to the treatment of heart failure. Pharmacol Ther 2005; 105:211–228.

5 Yoon YS, Johnson IA, Park JS, et al. Therapeutic myocardial angiogenesis with vascular endothelial growth factors. Mol Cell Biochem 2004; 264:63–74.

6 Graham RM, Bishopric NH, Webster KA. Gene and cell therapy for heart disease. IUBMB Life 2002; 54:59–66.

7 Liu X, Pachori AS, Ward CA, et al. Heme oxygenase-1 (HO-1) inhibits postmyocardial infarct remodeling and restores ventricular function. FASEB J 2006; 20:207–216.

8 Elrod JW, Greer JJ, Bryan NS, et al. Cardiomyocyte-specific overexpression of NO synthase-3 protects against myocardial ischemia-reperfusion injury. Arterioscler Thromb Vasc Biol 2006; 26:1517–1523.

9 Wang Y, Gabrielsen A, Lawler PR, et al. Myocardial gene expression of angiogenic factors in human chronic ischemic myocardium: influence of acute ischemia/cardioplegia and reperfusion. Microcirculation 2006; 13:187–197.

10 Azuma J, Taniyama Y, Takeya Y, et al. Angiogenic and antifibrotic actions of hepatocyte growth factor improve cardiac dysfunction in porcine ischemic cardiomyopathy. Gene Ther 2006; 13(16):1206–1213.

11 Ferrarini M, Arsic N, Recchia FA, et al. Adeno-associated virus-mediated transduction of VEGF165 improves cardiac tissue viability and functional recovery after permanent coronary occlusion in conscious dogs. Circ Res 2006; 98:954–961.

12 Pleger ST, Remppis A, Heidt B, et al. S100A1 gene therapy preserves in vivo cardiac function after myocardial infarction. Mol Ther 2005; 12:1120–1129.

13 Fan GC, Ren X, Qian J, et al. Novel cardioprotective role of a small heat-shock protein, Hsp20, against ischemia/reperfusion injury. Circulation 2005; 111:1792–1799.

14 Sugano M, Hata T, Tsuchida K, et al. Local delivery of soluble TNF-alpha receptor 1 gene reduces infarct size following ischemia/reperfusion injury in rats. Mol Cell Biochem 2004; 266:127–132.

15 Most P, Pleger ST, Volkers M, et al. Cardiac adenoviral S100A1 gene delivery rescues failing myocardium. J Clin Invest 2004; 114:1550–1563.

16 Jain M, DerSimonian H, Brenner DA, et al. Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction. Circulation 2001; 103:1920–1927.

17 Yoon YS, Wecker A, Heyd L, et al. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 2005; 115:326–338.

18 Shyu KG, Wang BW, Hung HF, et al. Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. J Biomed Sci 2006; 13:47–58.

19 Lee N, Thorne T, Losordo DW, Yoon YS. Repair of ischemic heart disease with novel bone marrow-derived multipotent stem cells. Cell Cycle 2005; 4:861–864.

20 Sakai T, Ling Y, Payne TR, Huard J. The use of ex vivo gene transfer based on muscle-derived stem cells for cardiovascular medicine. Trends Cardiovasc Med 2002; 12:115–120.

21 Leor J, Battler A, Kloner RA, Etzion S. Reprogramming cells for transplantation. Heart Fail Rev 2003; 8:285–292.

22 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410:701–705.

23 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.

24 Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001; 98:10344–10349.

25 Balsam LB, Wagers AJ, Christensen JL, et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004; 428:668–673.

26 Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428:664–668.

27 Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107:1395–1402.

28 Melo LG, Agrawal R, Zhang L, et al. Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene. Circulation 2002; 105:602–607.

29 Pachori AS, Melo LG, Zhang L, et al. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. J Am Coll Cardiol 2006; 47:635–643.

30 Liu X, Pachori AS, Ward CA, et al. Heme oxygenase-1 (HO-1) inhibits postmyocardial infarct remodeling and restores ventricular function. FASEB J 2006; 20:207–216.

31 Zhu HL, Stewart AS, Taylor MD, et al. Blocking free radical production via adenoviral gene transfer decreases cardiac ischemia-reperfusion injury. Mol Ther 2000; 2:470–475.

32 Agrawal RS, Muangman S, Layne MD, et al. Pre-emptive gene therapy using recombinant adeno-associated virus delivery of extracellular superoxide dismutase protects heart against ischemic reperfusion injury, improves ventricular function and prolongs survival. Gene Ther 2004; 11:962–969.

33 Sun M, Dawood F, Wen WH, et al. Excessive tumor necrosis factor activation after infarction contributes to susceptibility of myocardial rupture and left ventricular dysfunction. Circulation 2004; 110:3221–3228.

34 Akasaka Y, Morimoto N, Ishikawa Y, et al. Myocardial apoptosis associated with the expression of proinflammatory cytokines during the course of myocardial infarction. Mod Pathol 2006; 19:588–598.

35 Smith RS, Jr, Agata J, Xia CF, et al. Human endothelial nitric oxide synthase gene delivery protects against cardiac remodeling and reduces oxidative stress after myocardial infarction. Life Sci 2005; 76:2457–2471.

36 Brown M, McGuinness M, Wright T, et al. Cardiac-specific blockade of NF-kappaB in cardiac pathophysiology: differences between acute and chronic stimuli in vivo. Am J Physiol Heart Circ Physiol 2005; 289:H466–H476.

37 Misra A, Haudek SB, Knuefermann P, et al. Nuclear factor-kappaB protects the adult cardiac myocyte against ischemia-induced apoptosis in a murine model of acute myocardial infarction. Circulation 2003; 108:3075–3078.

38 Chen Z, Chua CC, Ho YS, et al. Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. Am J Physiol Heart Circ Physiol 2001; 280:H2313–H2320.

39 Matsui T, Tao J, del Monte F, et al. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. Circulation 2001; 104:330–335.

40 Miao W, Luo Z, Kitsis RN, Walsh K. Intracoronary, adenovirus-mediated Akt gene transfer in heart limits infarct size following ischemia-reperfusion injury in vivo. J Mol Cell Cardiol 2000; 32:2397–2402.

41 Jayasankar V, Woo YJ, Pirolli TJ, et al. Induction of angiogenesis and inhibition of apoptosis by hepatocyte growth factor effectively treats postischemic heart failure. J Card Surg 2005; 20:93–101.

42 Liao Z, Brar BK, Cai Q, et al. Cardiotrophin-1 (CT-1) can protect the adult heart from injury when added both prior to ischaemia and at reperfusion. Cardiovasc Res 2002; 53:902–910.

43 Kato K, Yin H, Agata J, et al. Adrenomedullin gene delivery attenuates myocardial infarction and apoptosis after ischemia and reperfusion. Am J Physiol Heart Circ Physiol 2003; 285:H1506–H1514.

44 Murriel CL, Churchill E, Inagaki K, et al. Protein kinase Cdelta activation induces apoptosis in response to cardiac ischemia and reperfusion damage: a mechanism involving BAD and the mitochondria. J Biol Chem 2004; 279:47985–47991.

45 Uchiyama T, Engelman RM, Maulik N, Das DK. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. Circulation 2004; 109:3042–3049.

46 Hochhauser E, Kivity S, Offen D, et al. Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice. Am J Physiol Heart Circ Physiol 2003; 284:H2351–H2359.

47 Ruetten H, Badorff C, Ihling C, et al. Inhibition of caspase-3 improves contractile recovery of stunned myocardium, independent of apoptosis-inhibitory effects. J Am Coll Cardiol 2001; 38:2063–2070.

48 Chandrashekhar Y, Sen S, Anway R, et al. Long-term caspase inhibition ameliorates apoptosis, reduces myocardial troponin-I cleavage, protects left ventricular function, and attenuates remodeling in rats with myocardial infarction. J Am Coll Cardiol 2004; 43:295–301.

49 Matsusaka H, Ide T, Matsushima S, et al. Targeted deletion of p53 prevents cardiac rupture after myocardial infarction in mice. Cardiovasc Res. 2006; 70(3):457–465.

50 Li Y, Takemura G, Kosai K, et al. Critical roles for the Fas/Fas ligand system in postinfarction ventricular remodeling and heart failure. Circ Res 2004; 95:627–636.

51 Su H, Joho S, Huang Y, et al. Adeno-associated viral vector delivers cardiac-specific and hypoxia-inducible VEGF expression in ischemic mouse hearts. Proc Natl Acad Sci USA 2004; 101:16280–16285.

52 Suzuki G, Lee TC, Fallavollita JA, Canty JM, Jr. Adenoviral gene transfer of FGF-5 to hibernating myocardium improves function and stimulates myocytes to hypertrophy and reenter the cell cycle. Circ Res 2005; 96:767–775.

53 Zhou L, Ma W, Yang Z, et al. VEGF165 and angiopoietin-1 decreased myocardium infarct size through phosphatidylinositol-3 kinase and Bcl-2 pathways. Gene Ther 2005; 12:196–202.

54 Melo LG, Pachori AS, Kong D, et al. Molecular and cell-based therapies for protection, rescue, and repair of ischemic myocardium: reasons for cautious optimism. Circulation 2004; 109:2386–2393.

55 Duan HF, Wu CT, Wu DL, et al. Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. Mol Ther 2003; 8:467–474.

56 Forte G, Minieri M, Cossa P, et al. Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. Stem Cells 2006; 24:23–33.

57 Askari A, Unzek S, Goldman CK, et al. Cellular, but not direct, adenoviral delivery of vascular endothelial growth factor results in improved left ventricular function and neovascularization in dilated ischemic cardiomyopathy. J Am Coll Cardiol 2004; 43:1908–1914.

58 Kawamoto A, Murayama T, Kusano K, et al. Synergistic effect of bone marrow mobilization and vascular endothelial growth factor-2 gene therapy in myocardial ischemia. Circulation 2004; 110:1398–1405.

59 Ninomiya M, Koyama H, Miyata T, et al. Ex vivo gene transfer of basic fibroblast growth factor improves cardiac function and blood flow in a swine chronic myocardial ischemia model. Gene Ther 2003; 10:1152–1160.

60 Tang YL, Tang Y, Zhang YC, et al. Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. J Am Coll Cardiol 2005; 46:1339–1350.

61 Kusano KF, Pola R, Murayama T, et al. Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. Nat Med 2005; 11:1197–1204.

62 Li TS, Hayashi M, Ito H, et al. Regeneration of infarcted myocardium by intramyocardial implantation of ex vivo transforming growth factor-beta-preprogrammed bone marrow stem cells. Circulation 2005; 111:2438–2445.

63 Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003; 9:1195–1201.

64 Tang YL, Zhao Q, Qin X, et al. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005; 80:229–236; discussion: 236–237.

65 Gnecchi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006; 20:661–669.

66 Iwakura A, Shastry S, Luedemann C, et al. Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. Circulation 2006; 113:1605–1614.

67 Shintani S, Kusano K, Ii M, et al. Synergistic effect of combined intramyocardial CD34+ cells and VEGF2 gene therapy after MI. Nat Clin Pract Cardiovasc Med 2006; 3(suppl 1):S123–S128.

68 Moscoso I, Centeno A, Lopez E, et al. Differentiation "in vitro" of primary and immortalized porcine mesenchymal stem cells into cardiomyocytes for cell transplantation. Transplant Proc 2005; 37:481–482.

69 Abbott JD, Huang Y, Liu D, et al. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004; 110:3300–3305.

70 Oh H, Taffet GE, Youker KA, et al. Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. Proc Natl Acad Sci USA 2001; 98:10308–10313.

71 Pasumarthi KB, Field LJ. Cardiomyocyte cell cycle regulation. Circ Res 2002; 90:1044–1054.

72 Shiraishi I, Melendez J, Ahn Y, et al. Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. Circ Res 2004; 94:884–891.

73 Rota M, Boni A, Urbanek K, et al. Nuclear targeting of Akt enhances ventricular function and myocyte contractility. Circ Res 2005; 97:1332–1341.

74 Lanza R, Moore MA, Wakayama T, et al. Regeneration of the infarcted heart with stem cells derived by nuclear transplantation. Circ Res 2004; 94:820–827.

75 Kizana E, Ginn SL, Allen DG, et al. Fibroblasts can be genetically modified to produce excitable cells capable of electrical coupling. Circulation 2005; 111:394–398.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

20 CHAPTER 20

Cardiac stem cells and the failing heart

Piero Anversa, MD, *Konrad Urbanek,* MD, *Claudia Bearzi,* PhD, *Antonella De Angelis,* PhD, *& Marcello Rota,* PhD

Introduction

The recognition that the heart possesses a stem cell compartment that can regenerate myocytes and coronary vessels demonstrates that it is possible to reconstitute dead myocardium after infarction, to repopulate the hypertrophic decompensated heart with new better functioning myocytes and vascular structures, and perhaps to reverse ventricular dilation and wall thinning, restoring the physiological and anatomical characteristics of the normal heart. This hypothesis is supported by the identification of resident cardiac stem cells (CSCs) in the mammalian heart and the demonstration that CSCs can be isolated and expanded *in vitro* (see also Chap 9). These cells are self-renewing, clonogenic, and multipotent, and thereby capable of differentiating into cardiomyocytes and coronary vascular cells. Additionally, the local injection of CSCs in infarcted animals repairs the necrotic myocardium by forming functionally competent myocytes, coronary arterioles, and capillary structures. These observations have changed the perennial view of the heart as a postmitotic organ and have formed the basis of a new paradigm in which multipotent CSCs are implicated in the normal turnover of myocytes, endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts.

The appreciation that the heart is a dynamic organ constantly renewing its cell populations has generated great enthusiasm in the scientific and clinical communities. Understanding the mechanisms of cardiac homeostasis would offer the extraordinary opportunity to potentiate this naturally occurring process and promote myocardial regeneration following tissue injury. Hypothetically, stimulation of CSCs is unlikely to induce malignant neoplasms since they are an extremely rare form of cardiac pathology. Also, CSCs should be more effective in making new myocardium than stem/progenitor cells from other organs including the bone marrow. CSCs are programmed to create heart muscle and, on activation, can rapidly engender parenchymal cells and coronary vessels, possibly rescuing the failing heart.

However, the field of regenerative cardiology is in its infancy and great caution has to be exercised in the implementation of this form of cellular therapy in human beings before we have obtained the basic information concerning the ability of CSCs to migrate, divide, and differentiate. Similarly, ischemic and nonischemic pathologic states and the duration and severity of the disease may have profound implications on the function of the CSC pool. In addition, the age and gender of the patients have to be regarded as critical variables of CSC growth and lineage commitment. Unfortunately, there is no good animal model that can be employed to collect such information that is of tremendous importance in the application of CSC treatment to the patient population.

This chapter reviews current knowledge and strategies relevant to the potential future treatment of patients according to their disease history, age, sex, and clinical conditions. Ultimately, CSC therapy will have to be defined on an individual

basis to maximize the efficacy of this novel approach for the management of heart failure in each case.

Cell therapy of the failing heart

An important goal of stem cell therapy is to identify whether a category of CSCs is better set for the regeneration of both myocytes and coronary vasculature or whether distinct classes of CSCs differentiate predominantly in myocytes or in coronary vessels. Moreover, the rates of growth and maturation may differ among CSC populations in a manner that is currently unknown [1]. This information is crucial for the preparation of the "best" mixture of cells to be administrated in the treatment of specific myocardial diseases. Defects in coronary perfusion of the hypertrophied heart may benefit more from the expansion of the coronary bed [2], while idiopathic dilated cardiomyopathy may require a prevailing formation of myocytes to increase wall thickness and decrease chamber diameter [3]. However, ischemic myocardial injury necessitates both parenchymal cells and coronary vessels to rebuild the lost myocardium. In the presence of massive infarcts, regeneration has to occur rapidly to reduce the infarct to a size that is compatible with life [4].

Equally relevant is to determine whether the use of CSCs is preferable to the use of cells already committed to the myocyte, EC and SMC lineages or whether a pool of CSCs and partially differentiated cells provides a greater and faster regenerative response. A mixture of cells with clearly defined properties, but with a high potential for proliferation, may be more effective in reconstituting the dead myocardium after infarction than is an undifferentiated cell population. In addition, this pool may be more efficacious in replacing the poorly contracting myocytes of the chronically failing heart with younger, better functioning cells and a vascular supply. Under this setting, coronary reserve is impaired and minimal coronary vascular resistance is increased [5]. Thus, new myocytes, resistance arterioles, and capillary profiles may be rapidly developed to improve tissue oxygenation and cardiac pump function. Figure 20.1 illustrates graphically the problems at hand and the directions that may have to be followed to acquire better knowledge of the complexity of CSC therapy for human disease.

A third critical problem is to establish whether these strategies, which may lead to the repair of the acutely damaged heart, are equally effective, less effective, or completely ineffective in the regeneration of chronic scarred myocardium [6]. Our current understanding of the actual strengths and limits of the therapeutic potential of CSCs is primitive at best. The biology of CSCs, in terms of their growth properties and long-term repopulating ability *in vivo*, need to be determined for the recognition of the most primitive cell in the myocardium. These issues are important for the characterization of human CSCs whose applicability may dramatically advance the field of cellular therapy for the failing heart. Questions have been raised and protocols have been developed to impact on the future directions of regenerative cardiology. Negative experimental findings resulting from differences in protocols, methodological shortcomings, and suboptimal techniques for the analysis of myocardial repair have muddled the field of cell therapy and cardiac repair [7, 8], creating discomfort in the patient population and in the scientific–clinical community. Therefore, the promoters of the field of stem cell therapy have to acknowledge these recent findings, repeat some of the work, and provide unequivocal answers to the criticisms. At the same time, they have to continue to search for new and more innovative strategies for the reconstitution of the severely decompensated heart.

Cardiac stem cells

In the last few years, several laboratories have identified different subsets of progenitor cells in the myocardium [9]. Although these observations have promoted a revolution in basic cardiovascular science and clinical cardiology, the methodological approach has not been consistent among groups, making comparisons between progenitor cell categories extremely complex. It is important to reiterate the fundamental behavior of stem cells and the need to adhere to definitions. In fact, whether the several progenitor cell types described in the heart represent distinct populations remains questionable. Stem cells have been known for quite some time, but there is little understanding of the importance of stem cell surface antigens in the growth and differentiation potential of these cells [1, 10]. These

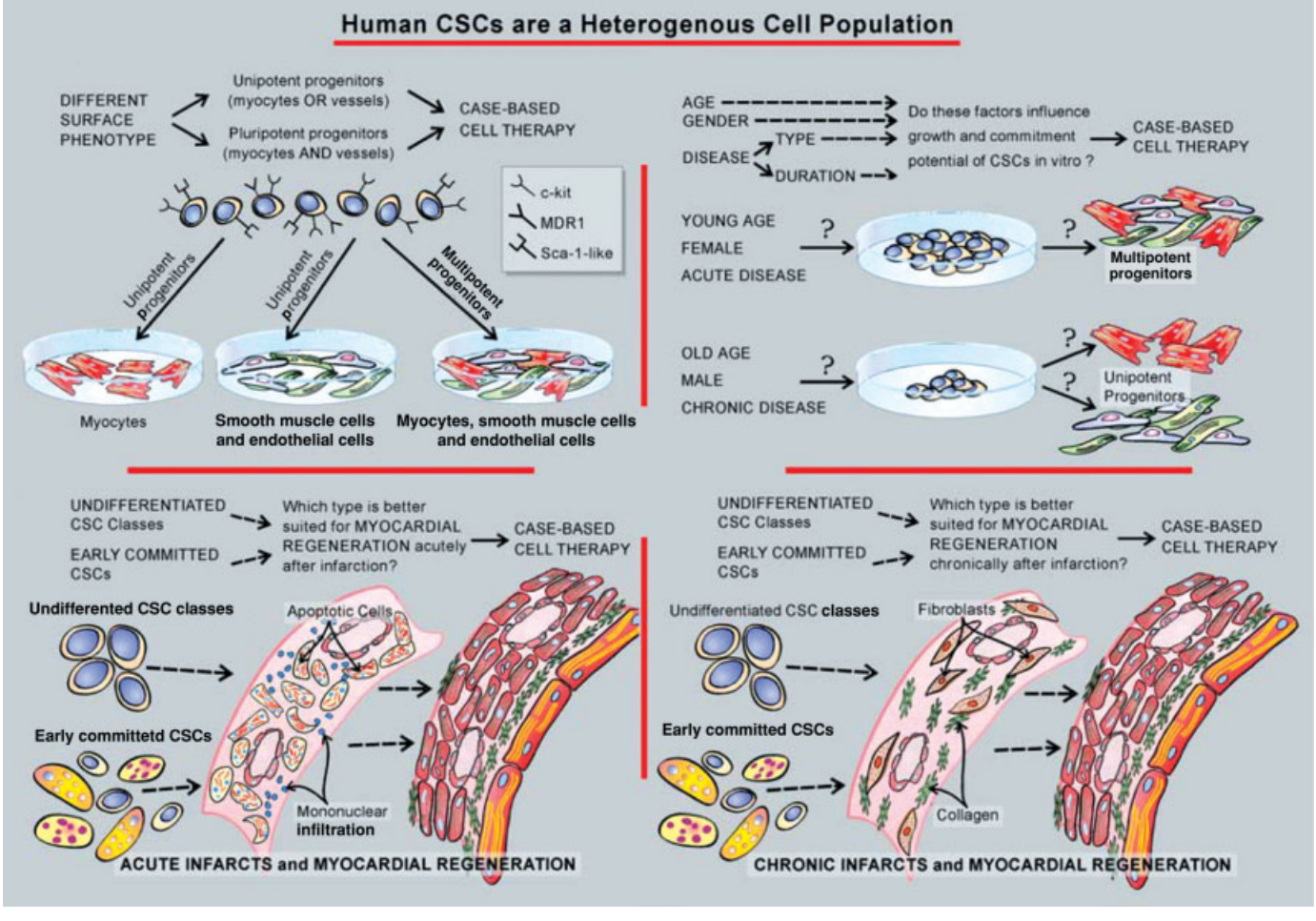


Figure 20.1 CSC properties. The growth properties of human CSC classes *in vitro* are summarized in the upper left panel, while the effects of age, gender, type, and duration of the disease on these *in vitro* parameters are shown in the upper right panel. Whether undifferentiated cells or a mixture of undifferentiated and partially committed cells have a distinct regenerative potential of the acutely infarcted or chronically infarcted heart are depicted in the lower two panels.

limitations are not restricted to the heart and the CSCs but apply to stem cells in all self-renewing organs, including the bone marrow. Moreover, the surface antigens c-kit, MDR1, or Sca-1 are present in cells undergoing lineage differentiation, complicating recognition of the actual primitive cells in the population [9]. The classification below offers a framework that reflects the studies done in the characterization of hematopoietic stem cells and their progeny and constitutes a reasonable basis for comparison.

CSCs are lineage-negative primitive cells expressing only the surface antigens c-kit, MDR1, and Sca-1 protein alone or in combination. CSCs are negative for transcription factors present in cardiac cells, GATA-4 and GATA-5; myocytes, Nkx 2.5 and MEF2C; ECs, Ets1 and Erg1; SMCs, GATA-6; skeletal muscle cells, MyoD, myogenin, and Myf5; and hematopoietic cells, GATA-2 and GATA-3. CSCs are also negative for cytoplasmic proteins specific for myocytes (nestin, desmin, cardiac myosin heavy chain, α-sarcomeric actin, connexin 43, and N-cadherin), ECs (CD34, CD31, von Willebrand factor, and flk1), SMCs (TGFβ1 receptor, flk1, and α-smooth muscle actin), neural cells (MAP1b, neurofilament 200, and GFAP), and hematopoietic cells (CD45, CD45RO, CD8, CD20, and TER 119). *Progenitor cells* express stem cell antigens and transcription factors of cardiac cell lineages or, more specifically, of myocytes, ECs, and SMCs. Progenitor cells do not express specific cytoplasmic proteins. *Precursor cells* exhibit stem cell antigens in combination with cytoplasmic proteins typical of myocytes, ECs, or SMCs.

Fluorescence-activated cell sorting (FACS) analysis is extremely helpful in separating stem cells in various categories according to the expression of one or more surface epitopes [11], but the distinction between undifferentiated and early committed cells necessitates caution. More sophisticated analyses of nuclear and cytoplasmic proteins by FACS require fixation of the cells to make them permeable and amenable to the detection of intracellular components. However, the viability of the cells is lost precluding any subsequent *in vivo* or *in vitro* study. Similar problems are encountered by immunocytochemistry that also demands cell fixation before the antibody or a cocktail of antibodies is utilized to determine the stemness or commitment of the cells.

So far, experimental studies and clinical trials have employed rather heterogeneous bone marrow cell preparations in an attempt to regenerate dead myocardium after infarction. With a few exceptions [7, 8], the injection of bone marrow cells has resulted in a consistent improvement in function of the infarcted heart in both animals and humans [12–15]. However, these reports have not answered the critical question whether one bone marrow cell has a more powerful therapeutic efficacy than another. It is hard to envision that any cell of bone marrow origin can form new myocardium with functionally competent myocytes and coronary vessels. In the majority of cases, the administrated cells were not characterized and, most likely, represented a combination of therapeutically effective and noneffective cells. Importantly, cells with distinct epitopes were not tested. Similarly, CSCs positive for c-kit or Sca-1 have been employed in rodents after infarction but no comparison was made with other CSC classes [10, 16]. Thus, the identity of the most appropriate cell for the management of heart failure remains essentially unknown.

The c-kit$^+$ CSC was the first stem cell identified in the rat heart [10]. More recent studies have indicated that Sca-1$^+$ CSCs are the predominant stem cell population in the mouse heart [16, 17]. According to these observations, Sca-1$^+$ CSCs are 100- to 700-fold more frequent than c-kit$^+$ CSCs. Similarly, MDR1$^+$ CSCs have been isolated and found to represent a small fraction of all CSCs in the mouse heart [18]. Comparable results with regard to hematopoietic stem cells have been reported in the mouse bone marrow. Quantitative data from our laboratory have demonstrated that in the mouse heart there is one CSC per ~30,000 myocardial cells: 63% of all CSCs possess the three stem cell antigens (c-kit, MDR1, Sca-1); 22% possess two stem cell antigens, and 15% possess only one. Approximately 5% each of all CSCs express exclusively c-kit, MDR1, or Sca-1 [19]. However, this work did not resolve the issue whether the variability in the distribution of these surface markers actually reflects cells with distinct functional import. Some information can be obtained from the available transgenic mice: the Sca-1$^{-/-}$ mouse and the MDR1$^{-/-}$ mouse [20, 21] have essentially a normal phenotype with modest defects of the hematopoietic system and other organs. Recently, the Isl1 transcription factor has been

shown to be associated with a cell population that conditions heart morphogenesis in the mouse embryo [22]. Defects in the development of the right ventricle, atria, and outflow tract have been linked to the homozygous deletion of Isl1. Conversely, the expression of Isl1 in primitive cells clustered in the niches or scattered throughout the atrial and ventricular myocardium of the adult mouse heart is, at best, extremely rare. In our research, we have not found even a single example. Similarly, no Isl1$^+$ cells have been detected in the acutely and chronically failing human heart (Figure 20.2). Although these initial results do not exclude that some Isl1$^+$ cardiomyoblasts may be present in the adult myocardium, the functional impact of these cells on cardiac pathology, if it exists, is debatable.

In contrast to the deletion of Sca-1 or MDR1, mutation of the c-kit receptor in the W/W^V mouse has profound effects on the phenotype of the animal. The W/W^V mouse has a spontaneous point mutation in one allele (W^V) coupled with the deletion of a large sequence of amino acids in the other allele (W) of the c-kit receptor [23]. The homozygous W/W mouse dies shortly after birth, while the heterozygous W/W^V has defects of the hematopoietic system with the development of anemia, melanocytes with loss of skin pigmentation, and mastocytes with altered immunoresponse. The W/W^V mouse reaches adulthood and the heart shows at 8–9 months alterations in cardiac anatomy and function that precede the overt manifestations of anemia. Thus, mutation of the c-kit receptor attenuates the growth of CSCs and their commitment to the myogenic lineage resulting in a reduction of myocyte formation, accumulation of senescent myocytes, and alterations in ventricular performance and cardiac anatomy [24]. Conversely, the numerical density and the total length of arterioles and capillaries in the ventricle are preserved. In view of the multipotentiality of c-kit$^+$ CSCs *in vitro* and *in vivo*, the contribution of these cells to the formation of ECs and SMCs organized in coronary vessels was compensated in W/W^V mice by other CSC classes. In this model, c-kit$^+$ CSCs may play a major role in myocyte regeneration and a lesser role in vasculogenesis. This notion is consistent with the ability of rat clonogenic c-kit$^+$ CSCs to extensively repair the infarcted heart following permanent coronary artery occlusion [10] or ischemia-reperfusion injury [25] in syngeneic animals. Conversely, Sca-1$^+$ CSCs have a little impact on the repair of the heart following ischemic damage [16].

On this basis, the possibility is advanced that c-kit$^+$ CSCs are the predominant primitive cell population responsible for myocyte regeneration in the adult heart, while their involvement in the formation of coronary arterioles and capillary structures is less critical for the preservation of myocardial perfusion. Therefore, the expression of distinct stem cell epitopes has a functional counterpart that can be exploited to improve myocardial repair. However, the needs of the overloaded failing heart vary with the etiology of the initiating event, the stage of the disease process, and the age and sex of the patient. Conditions exist in which the formation of large coronary vessels and resistance arterioles is more important than that of myocytes. Conversely, other situations require a larger number of myocytes than coronary vessels or a well-balanced mixture of the two. Information concerning the growth

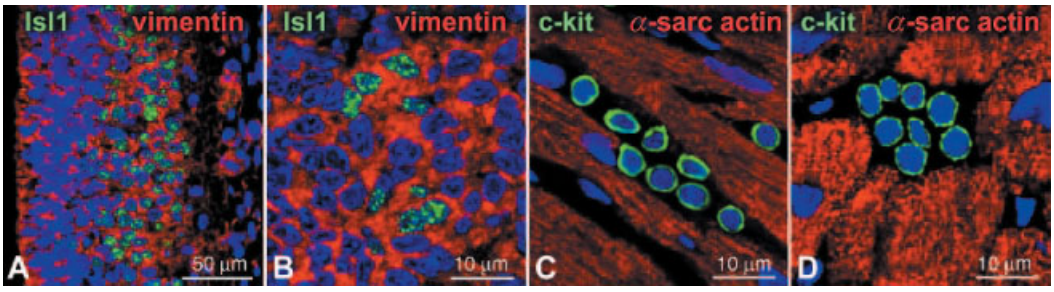


Figure 20.2 c-kit and Isl1 cells in the myocardium. Isl1$^+$ cells are illustrated in the neural crest of a 13-day-old rat embryo at both (A) low and (B) higher magnification. Isl1$^+$ cells are not found (C) in the adult normal rat heart and (D) in the failing human heart, which have c-kit-positive cells.

characteristics of CSC classes is critical if less empirical and carefully planned strategies are to be implemented for the successful rebuilding of the decompensated heart.

CSCs and human heart failure

Coronary artery disease and systemic hypertension are the major causes of congestive heart failure [2, 6, 26], but myocardial infarction and high blood pressure lead to heart failure by mechanisms that are initially different, remain distinct during the progression of the pathologic processes, and may become comparable only in the late evolution to terminal failure [26, 27]. The post-infarcted heart typically shows cavitary dilation, wall thinning, and an increase in both diastolic and systolic wall stress [28]. In contrast, systemic hypertension is characterized by thickening of the wall and an increase in wall thickness-to-chamber radius ratio. The hypertensive heart is faced initially with an increased systolic load, and only in the advanced stage, when decompensation develops, does an abnormal elevation in left ventricular end-diastolic pressure together with thinning of the wall and chamber dilation becomes apparent [27]. Defects in coronary blood flow are present in both pathological states, but the abnormalities in myocardial perfusion are more prominent in hypertensive hypertrophy than in the postinfarcted heart. Similarly, the phenotypic architecture and loading of the heart vary significantly in ischemic heart disease, hypertension, idiopathic dilated cardiomyopathy [3, 26], and in the unsuccessful repair of a valvular defect with persistence of ventricular dysfunction and myocardial hypertrophy [28]. Whether this difference in cardiac pathology impacts differently on CSCs is unclear, but is an important question.

The chronic evolution of these disease processes is characterized by the formation of multiple sites of replacement fibrosis across the myocardium, although they tend to accumulate in the lower midregion and subendocardial layer of the ventricular wall. This phenomenon is typically seen in ischemic heart disease. In the progression of ischemic cardiomyopathy, in fact, the size of the infarct is not a good predictor of the short-, mid-, and long-term outcome of the disease. Negative remodeling and accumulation of damage in the surviving myocardium become the critical determinants of cardiac dysfunction and terminal failure. The number of acute events differ in patients and, depending on the nature of the damage, segmental losses of myocardium require therapeutic approaches that are by far more complex than those for acute infarcts or small foci of tissue injury scattered in the myocardium. Observations in humans acutely after infarction [29, 30] or sustained pressure overload [31] indicate that the degree of differentiation that newly formed parenchymal cells acquire when they are clustered together in a large area of lost tissue is markedly distinct from that of regenerated myocytes dispersed throughout the viable myocardium in close proximity to mature preexisting cells. In the former case, myocytes are small and the myofibrils are rare and occupy only a minimal portion of the cytoplasm resembling fetal–neonatal cells. In the latter case, the regenerated myocytes are indistinguishable from the adjacent muscle cells and exhibit an adult phenotype.

This is an attractive biological problem that raises some interesting questions concerning the microenvironment and the cross talk between differentiated and developing myocytes. It is difficult to foresee how the etiology of heart failure and the stage of the disease alter the CSC pool, and thereby the ability of the heart to respond to the worsening of cardiac performance promoting formation of parenchymal cells and coronary vessels. Moreover, how the myocytes and the coronary vasculature and microvasculature are generated in an organized orderly manner is unknown. Similarly, whether the vascular scaffolding is needed for a proper development of the myocyte compartment or the muscle mass constitutes the framework that conditions the insertion of the coronary bed is currently unclear.

The recognition that the expression of a single stem cell antigen or their combination is linked to the formation of a preferential cardiac cell progeny or progenies may allow us to develop very powerful and novel strategies for the regeneration of myocytes and coronary vasculature and the restoration of cardiac performance in the failing heart. According to need, a new tool may become available for the predominant formation of myocytes and/or coronary vessels. To gain some insights into the functional implications that CSCs with different surface antigens may have in myocardial growth in large

mammals, CSCs were isolated from the dog heart and characterized [1]. Clones were developed from CSCs with the three epitopes and from CSCs with one epitope each (Figure 20.3).

Clones developed from single CSCs expressing c-kit, MDR1, or Sca-1-like only maintained the same phenotype and never changed the expression of the initial epitope in the clones and in the subclones. The size of the clones and subclones, developed at the same time under identical *in vitro* conditions, varied significantly when the clonogenic cells expressed different epitopes. c-kit$^+$ clones were significantly larger than MDR1$^+$, Sca-1-like$^+$, and c-kit–MDR1–Sca-1-like$^+$ clones. Thus, the expression of

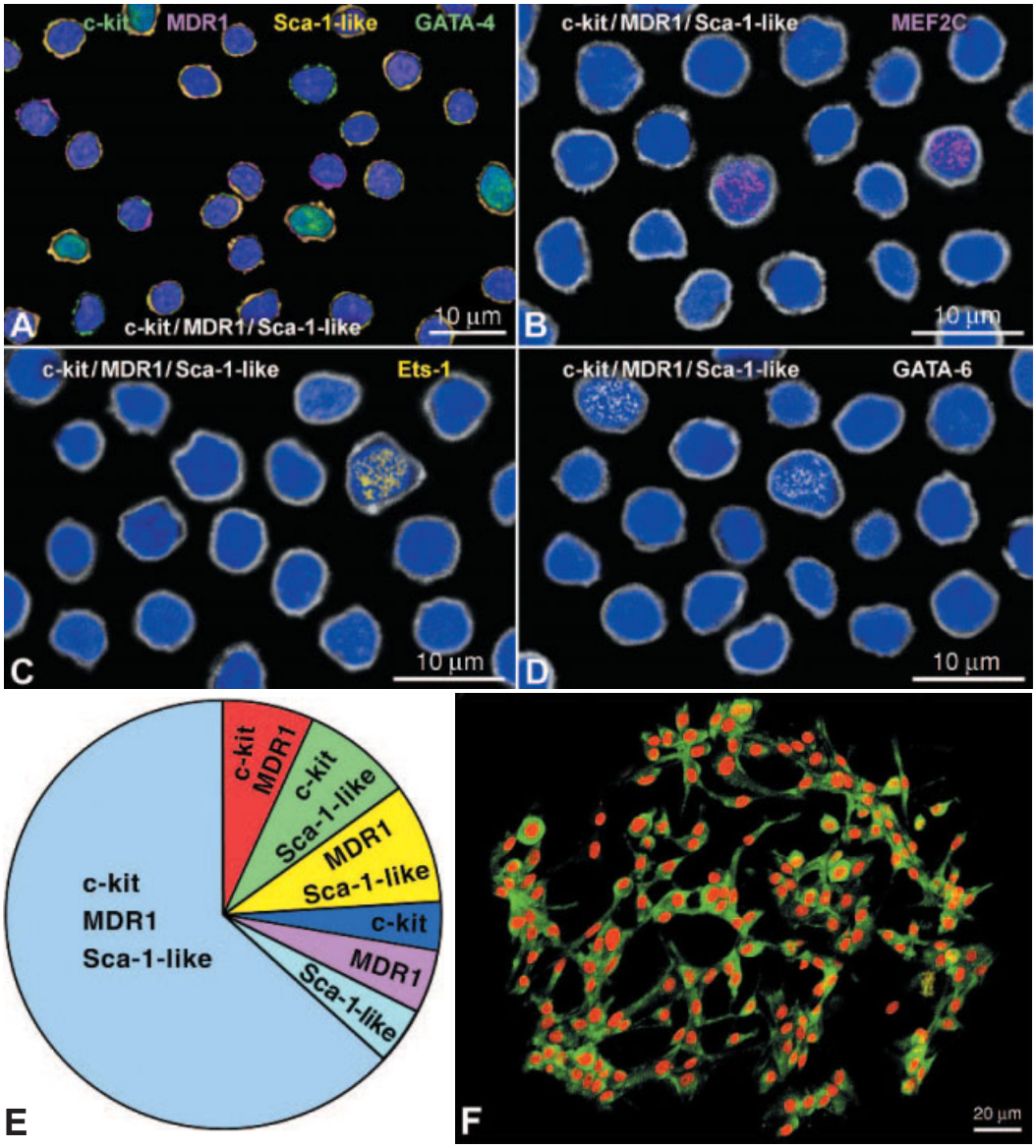


Figure 20.3 (A–E) Localization and relative proportions of stem cell antigens in cardiac primitive and early committed cells. (F) A clone developed from a single c-kit$^+$ cell. (G–J) Clone-derived differentiated cells. In the bar graph (K), M = myocytes; SMC = smooth muscle cells; and EC = endothelial cells. Panels (A–D) and (F–J) adapted from (cont.).

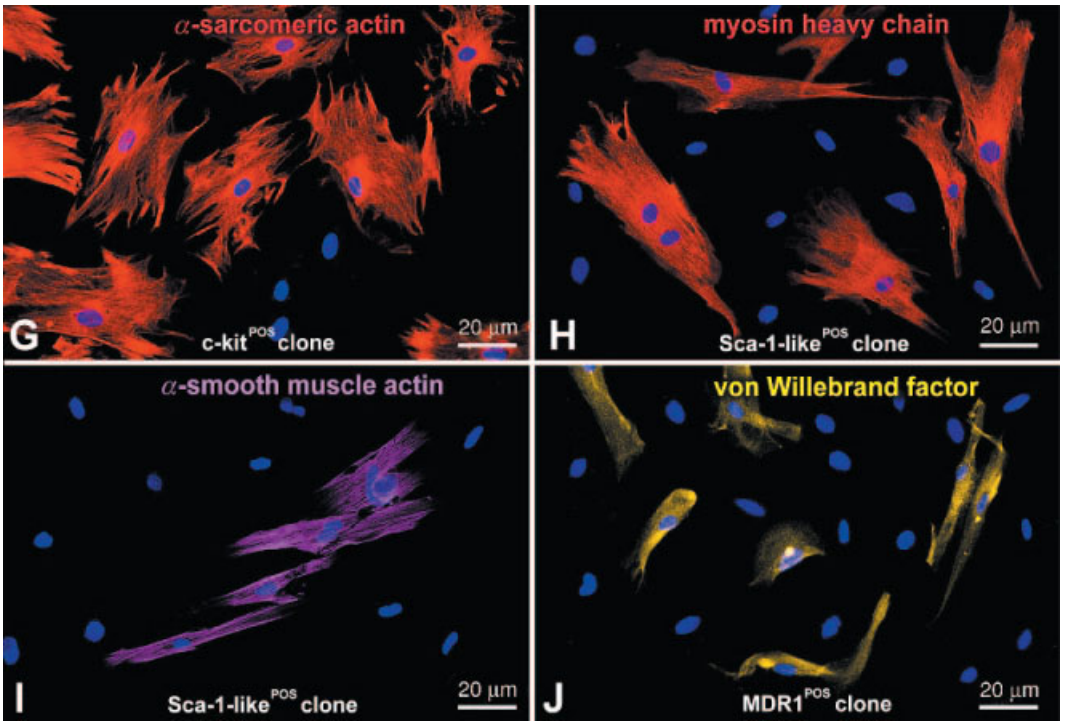


Figure 20.3 (*Continued*)

c-kit appears to be the major determinant of the self-renewing properties of CSCs. When the same number of cells from each distinct clone was placed in a differentiating medium, the total number of committed cells differed whether they were generated from c-kit$^+$, MDR1$^+$, or Sca-1-like$^+$ clonogenic cells or from c-kit–MDR1–Sca-1-like$^+$ cells. Over a period of 4 weeks, 11×10^5, 4×10^5, 2×10^5 cells were formed from c-kit$^+$, MDR1$^+$, and Sca-1-like$^+$ cells, respectively. c-kit–MDR1–Sca-1-like$^+$ clonogenic cells created 2×10^5 cells. Therefore, the ability of clonogenic c-kit$^+$ cells to create myocardial cells was much greater than that of MDR1$^+$, Sca-1-like$^+$, and c-kit–MDR1–Sca-1-like$^+$ clonogenic cells. Although different clonogenic cells generated different numbers of myocardial cells, they gave rise to similar proportion of cardiac cell lineages: ~30–32% myocytes, ~22–29% SMCs, ~6–12% ECs, ~12–18% fibroblasts, and ~17–24% undifferentiated cells. Whether the equivalent classes of human CSCs will react and grow in a similar manner is difficult to predict. Nevertheless, these possibilities have to be explored. If confirmed, this information will be highly relevant to the implementation of cellular therapy in patients. CSC treatment will be programmed according to the pathologic state of the patient who will be treated with an autologous specific CSC expanded *in vitro* to correct a myocyte deficiency, a vascular defect, or both.

Conclusions

Effort has been made to isolate CSCs from small samples of myocardial tissue obtained from patients undergoing cardiac surgery and extracorporeal circulation. Discarded samples such as the atrial appendage that was removed for the implantation of the pump were utilized for this purpose. A methodology was developed by which cardiac progenitor cells were isolated from the myocardium and expanded *in vitro* for a subsequent sorting, characterization, and cloning protocol [32]. To document that the *in vitro* expanded human CSCs are capable of differentiating, reaching functional competence, and repairing the damaged myocardium, infarcts were produced in immunodeficient mice and

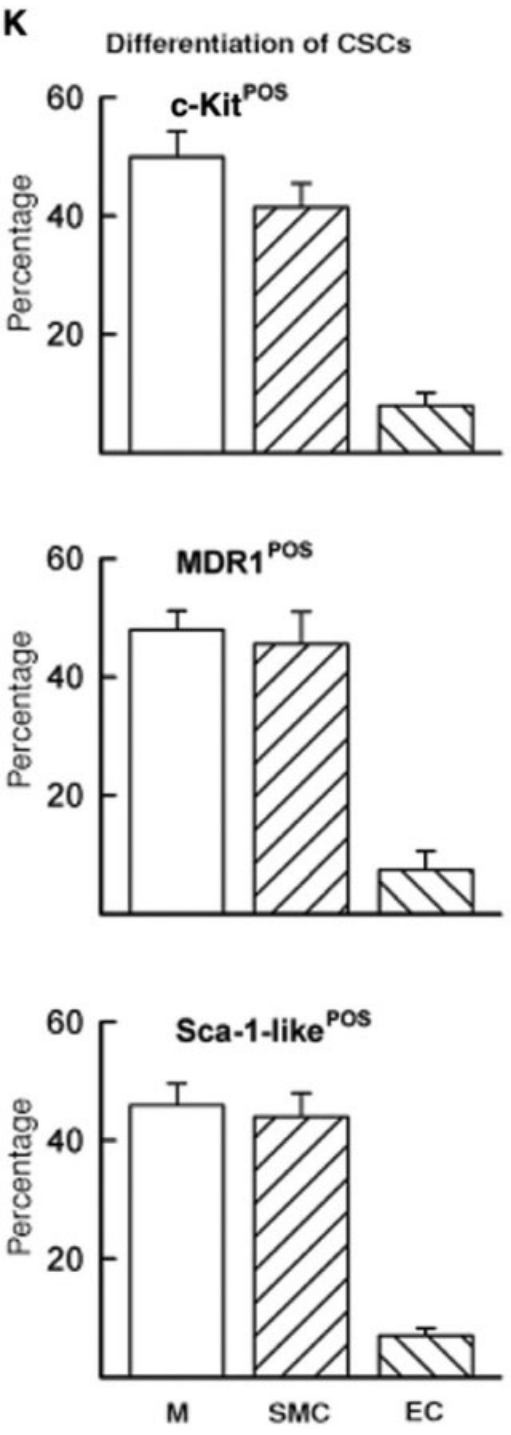


Figure 20.3 (*Continued*)

immunosuppressed rats and the human CSCs were injected in the contracting myocardium bordering the infarct shortly after coronary artery ligation. The human CSCs regenerated human myocytes and coronary vessels, reducing the magnitude of ischemic injury and improving the performance of the infarcted heart. These data indicate that therapeutic strategies with CSCs are feasible and might be relevant to the management of human disease.

Acknowledgment

This work was supported by NIH.

References

1 Linke A, Muller P, Nurzynska D, et al. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent, and regenerate infarcted myocardium, improving cardiac performance. Proc Natl Acad Sci USA 2005; 102:8966–8971.
2 Redfield MM. Heart failure—an epidemic of uncertain proportions. N Engl J Med 2002; 347:1442–1444.
3 Beltrami CA, Finato N, Rocco M, et al. The cellular basis of dilated cardiomyopathy in humans. J Mol Cell Cardiol 1995; 27:291–305.
4 Solomon SD, Zelenkofske S, McMurray JJV, et al. For the valsartan in acute myocardial infarction trial (VALIANT) investigators. Sudden death in patients with myocardial infarction and left ventricular dysfunction, heart failure, or both. N Engl J Med 2005; 352:2581–2588.
5 Rakusan K, Flanagan MF, Geva T, et al. Morphometry of human coronary capillaries during normal growth and the effect of age in left ventricular pressure-overload hypertrophy. Circulation 1992; 86:38–46.
6 Jessup M, Brozena S. Heart failure. N Engl J Med 2003; 348:2007–2018.
7 Balsam LB, Wagers AJ, Christensen JL, et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004; 428:668–673.
8 Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428:664–668.
9 Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 2006; 113:1451–1463.
10 Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.
11 Tjonnfjord GE, Steen R, Veiby OP, et al. Haemopoietic progenitor cell differentiation: flow cytometric

assessment in bone marrow and thymus. Br J Haematol 1995; 91:1006–1016.
12 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 420:701–705.
13 Yoon YS, Wecker A, Heyd L, et al. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 2005; 115:326–338.
14 Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005; 85:1373–1416.
15 Wollert KC, Drexler H. Clinical applications of stem cells for the heart. Circ Res 2005; 96:151–163.
16 Oh H, Bradfute SB, Gallardo TD, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 2003; 100:12313–12318.
17 Matsuura K, Nagai T, Nishigaki N, et al. Adult cardiac Sca-1 positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004; 279:11384–11391.
18 Pfister O, Mouquet F, Jain M, et al. CD31– but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 2005; 97:52–61.
19 Urbanek K, Rota M, Cascapera S, et al. Cardiac stem cells possess growth factor-receptor systems that following activation regenerate the infarcted myocardium improving ventricular function and long-term survival. Circ Res 2005; 97:663–673.
20 Schinkel AH, Mayer U, Wagenaar E, et al. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc Natl Acad Sci USA 1997; 94:4028–4033.
21 Ito CY, Li CY, Bernstein A, et al. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood 2003; 101:517–523.
22 Laugwitz KL, Moretti A, Lam J. et al. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005; 433:585–587.
23 Reith AD, Rottapel R, Giddens E, et al. W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. Genes Dev 1990; 4:390–400.
24 Barlucchi L, Baker M, Padin-Iruegas ME, et al. Mutation of the c-kit receptor in cardiac progenitor cells results in decompensated eccentric hypertrophy following aortic stenosis in mice. Circulation 2003; 108(suppl IV):IV–289.
25 Dawn B, Stein AB, Urbanek K, et al. Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. Proc Natl Acad Sci USA 2005; 102:3766–3771.
26 Beltrami CA, Finato N, Rocco M, et al. Structural basis of end-stage failure in ischemic cardiomyopathy in humans. Circulation 1994; 89:151–163.
27 Olivetti G, Melissari M, Balbi T, et al. Myocyte cellular hypertrophy is responsible for ventricular remodelling in the hypertrophied heart of middle aged individuals in the absence of cardiac failure. Cardiovasc Res 1994; 28:1199–1208.
28 Anversa P, Olivetti G. Cellular basis of physiological and pathological myocardial growth. In Handbook of Physiology, Section 2: The Cardiovascular System: The Heart, Vol 1. New York: Oxford University Press, 75–144, 2002.
29 Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001; 344:1750–1757.
30 Urbanek K, Torella D, Sheikh F, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 2005; 102:8692–8697.
31 Urbanek K, Quaini F, Tasca G, et al. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci USA 2003; 100:10440–10445.
32 Bearzi C, Rota M, Tillmans J, et al. Human cardiac progenitor cells regenerate cardiomyocytes and coronary vessels repairing the infarcted myocardium. Circulation 2004; 112:II–129.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

21

CHAPTER 21

Myocardial infarction: risks and hope

Marc A. Pfeffer, MD, PhD

Introduction

The person experiencing a myocardial infarction is making an immediate and profound statement "please help me – I have a systemic disease called atherosclerosis which is at present leading to the death of some of my precious cardiomyocytes". Both the health care and lay communities are well aware that this is an emergency situation requiring prompt medical attention. In the hyperacute phase, when regional myocardial ischemia is transitioning to cell death, the propensity for a fatal arrhythmia is so extreme that many of the deaths occur even before presentation to a hospital. Indeed, the major impetus for and the initial success of coronary care units resided in the ability to monitor cardiac rhythm and to employ direct electrical cardioversion for life-threatening and former life-ending ventricular arrhythmias.

Coronary care units

The development of coronary care units by concentrating expertise in the management of patients with myocardial infarction also had a profound influence on subsequent research and clinical accomplishments. Perhaps the next major phase of development of coronary care during its infancy was the realization that not all myocardial infarctions are equal. The clinical differentiation based on a hemodynamic characterization of systemic perfusion and the presence of pulmonary congestion provided one of the early risk stratification systems [1, 2]. With the incorporation of cardiac enzymes, later to become cardiac markers, into the clinical diagnosis of myocardial infarction, it soon became apparent that the extent of myocardial necrosis was highly variable [3]. Some infarcts were associated with barely detectable cardiac marker evidence of necrosis, whereas others resulted in extensive cell death. It was readily apparent that the patients with greater cardiac marker evidence of cell death were more likely to have a clinically complicated myocardial infarction including cardiogenic shock. The routine integration of biochemical measures of infarct size into clinical care set the stage for one of the most major paradigm shifting discoveries in clinical cardiology.

Myocardial salvage

Dr. Eugene Braunwald led a series of studies that delineated the determinants of oxygen consumption of the heart [4]. Myocardial ischemia became understood as an imbalance between the supply and demand of the myocardium for oxygen. A myocardial infarction was a consequence of prolonged ischemia leading to irreversible cell death. A temporal pattern was described for this cell death, beginning in the subendocardium and progressing as a "wavefront" to epicardial regions [5]. In experimental models of coronary occlusion, Braunwald, along with the late Peter Maroko and colleagues, demonstrated that by altering myocardial oxygen supply/demand during the first few hours following coronary artery occlusion, the extent of this progressive wavefront of cell death could be modified [6, 7]. This groundbreaking discovery that myocardial infarct size could be limited profoundly altered the management of patients with acute myocardial

infarction [8]. The concept of myocardial salvage provided the experimental rationale [9] for clinical trials that eventually demonstrated that prompt pharmacological (thrombolytics) [10, 11] and mechanical measures (primary coronary angioplasty) [12, 13] to restore coronary flow could limit infarct size, improve left ventricular function, and, most importantly, reduce the risk of death as well as other major nonfatal cardiovascular complications of a myocardial infarction.

The demonstration of an early and sustained survival benefit with the early introduction of thrombolytic therapy in patients with ST-segment elevation infarction along with the concurrent progress in percutaneous coronary interventions dramatically shifted the focus of acute coronary care into the "time equals myocytes" mode. Terms like *door to needle*, *door to balloon*, *open artery*, and *TIMI grade 3 flow* all became part of the everyday parlance for not just cardiologists and emergency department physicians, but also hospital administrators [14]. Indeed, the promptness of recognizing and treating a patient with an acute myocardial infarction in order to salvage myocardium has gone from concept to a quantifiable hospital performance measure.

Antiplatelet therapy

The role of platelet activation in contributing to thrombus formation, the interruption of blood flow during the acute phase of myocardial infarction, and risk of reinfarction was exquisitely well demonstrated in the 2 × 2 factorial design of ISIS II. In this landmark randomized, placebo-controlled clinical trial, significant survival benefits were individually achieved with the intravenous administration of the thrombolytic streptokinase as well as with the prompt use of oral aspirin [11]. Importantly, there was a clear additive reduction in risk of death in those randomized to receive both active therapies. Once a therapy is found effective, fine-tuning studies generally push the envelope to probe for additional potential benefits. Clopidogrel reduces platelet aggregation by inhibiting the adenosine diphosphate $P2Y_{12}$ receptor, a distinctly different mechanism than the irreversible inhibition of platelet cyclooxygenase produced by aspirin. Dual antiplatelet therapy (aspirin and clopidogrel) has been shown to be effective in several acute coronary syndrome settings and, most recently, was shown to result in further reductions in mortality without excessive bleeding risks when administered to patients with acute ST-elevation myocardial infarctions [15, 16].

Adjunctive therapy with an antithrombotic agent also reduces risk for subsequent atherothrombotic events during the acute phase. Evidence is accumulating that newer agents, such as low-molecular-weight heparin (enoxaparin) and a synthetic pentasaccharide (fondaparinux), offer clinical advantages over unfractionated heparin [17, 18]. The inherent risks of major hemorrhagic complications when administering multiple agents to reverse and block atherothrombosis underscores the complexities of acute coronary care and the need for highly experienced and specialized care in the acute phase of a myocardial infarction. Indeed, acute coronary care has evolved into a highly specialized aspect of cardiology involving the coordination of the sophisticated team of caregivers to promptly diagnose and optimally treat acute coronary syndromes to reduce the immediate risk of death and to salvage as much myocardium as possible.

Risk factor modification

There is an important transition in clinical management that generally coincides with the transfer of the patient from the acute coronary care unit to a monitored intermediate setting. By definition, these patients have survived the hyperacute phase of a myocardial infarction and have already been the recipients of therapies to reduce the extent of myocardial necrosis. This is a natural time to reassess prognosis, especially long-term risk, and to evaluate management options. Assessing risk is optimizing therapeutic opportunities, not assigning blame. Survivors of myocardial infarction, for the most part, can look forward to resuming a full and active lifestyle. However, what is clearly different is that they have now identified themselves as someone who not only has coronary artery disease but also had an acute event, which resulted in some loss of functional myocardium.

In assessing risk for future cardiovascular morbidity and mortality, it is essential to retrace premyocardial infarction risk factors. Although genetics

play a prominent role in cardiovascular (as well as other) risks, disavowing one's heritage is not an effective risk-modifying strategy. Advanced age, perhaps the most important risk factor, is also currently not modifiable.

Fortunately, there are several significant opportunities to lower subsequent risk. For smokers, there is no more important intervention than the lifestyle change of cigarette cessation. One of the important hospital performance measures for acute myocardial infarction care is the documentation that instructions regarding smoking cessation were appropriately administered. Presence of diabetes, whether known prior to the infarct or newly diagnosed, portends a 30–40% increased risk of all the major cardiovascular complications during the acute and chronic phase of a myocardial infarction [19]. Similarly, having hypertension prior to the infarct is associated with a greater risk of subsequent events, even if arterial pressure following the myocardial infarction is within the "normotensive range" [20, 21].

The importance of lipids levels, specifically high LDL cholesterol and low HDL cholesterol, as a modifiable risk factor has been underscored by multiple well-conducted, randomized, placebo-controlled clinical trials of statins. In both primary and secondary prevention trials, statins have proven to be well tolerated, safe, and effective in improving survival and reducing rates of atherosclerotic events [22]. Although it has long been known that patients with end-stage renal disease are among the highest risk cohort, more recent data have underscored that even more modest levels of renal impairment can greatly augment the cardiovascular risk following myocardial infarction [23]. With similar relative risk reductions, when used in primary and secondary prevention, the absolute benefit in lives saved and nonfatal events prevented by a statin is considerably greater in the higher risk postmyocardial infarction population [24].

A survivor of myocardial infarction is at a much higher risk for sudden death, sustaining a recurrent infarction, and/or the development of congestive heart failure than those with coronary artery disease who have not had an acute event. These risks are greatest in the weeks and first months following the initial event and remain above premyocardial infarction baseline risk even years after the acute event [25]. In the context of regenerative cell therapy, a current therapeutic target is to improve left ventricular function and thereby reduce the risk of developing chronic heart failure and its sequelae by augmenting or restoring contractile tissue. Concerns have been raised that malignant ventricular arrhythmias can be provoked by the imposition of a focus of cells that are not fully electromechanically integrated into the myocardium [26]. There is also no current reason to hypothesize that attempts to restore contractile tissue would reduce the risk of subsequent coronary atherothrombotic events. One preliminary report indicated an increased restenosis rate in patients who received direct intracoronary administration of cells mobilized with granulocyte colony-stimulating factor [27]. Therefore, a major potential therapeutic opportunity for regenerative cell-based therapies for patients experiencing a myocardial infarction would be to identify those who, despite current optimal treatment, remain at substantial risk for developing severe life limiting heart failure due to the loss of contractile tissue. Selection of these patients at highest risk for heart failure complications following myocardial infarction will be an important aspect of optimizing the risks/benefits of novel cell-based therapies [28]. Determining the appropriate inclusion characteristics of patients in future pivotal clinical trials will be critical and should be based on the pathophysiologic basis of postmyocardial infarction heart failure.

Risk of heart failure following myocardial infarction

By definition, the key difference in an individual prior to and following a myocardial infarction is that there has been a loss of contractile tissue as a consequence of the myocardial necrosis. The resultant left ventricular function is closely related to the magnitude of myocardial damage. Although generally assessed as left ventricular ejection fraction, more quantitative measures, such as end systolic or end diastolic volumes, the extent of noncontractile tissue assessed by akinesis and dyskinesis, or scar area as detected by late enhancement contrast using magnetic resonance imaging, can all be used to provide an important component of risk assessment for survivors of myocardial infarction. Regardless of the specific measure, the extent of damage and the

closely linked degree of left ventricular dysfunction are key determinants of subsequent risk of developing heart failure. Although the loss of myocardium is abrupt, it is not uncommon for the clinical appearance and recognition of sufficient clinical signs and symptoms of heart failure to occur years after the myocardial infarction [29, 30]. Those patients that go on to develop clinical heart failure postmyocardial infarction are at much higher risk of subsequent death, with median survivals estimated of only approximately 2–3 years.

Left ventricular remodeling following myocardial infarction

Animal studies provided mechanistic insights regarding the development of chronic heart failure following the acute loss of myocytes as a consequence of a myocardial infarction. As anticipated, following left coronary artery ligation there is an abrupt reduction in contractile function in the region no longer adequately perfused. Acutely, stroke volume and left ventricular ejection fraction may both be reduced in direct relation to the extent of the myocardial damage [31]. In clinical studies using serial echocardiography, a process of thinning and elongation of the noncontractile region of larger infarctions, termed infarct expansion, had been defined [32]. This initial distortion of the ventricular geometry often heralds a longer, more insidious process of ventricular enlargement [33, 34]. The late Dr. Janice Pfeffer led a series of studies that identified and characterized the process of left ventricular remodeling following myocardial infarction [34, 35]. She demonstrated that the enlargement of the left ventricular chamber went beyond just infarct expansion and was also a consequence of structural alterations in the border zone as well as the remote hyperfunctioning residual myocardium [35]. Although this global enlargement of ventricular chamber may initially provide some compensation to restore stroke volume and other aspects of pump function, the alterations in ventricular size and geometry could become self-sustaining and contribute to a progressive decline in ventricular function [36]. In effect, the enlarged chamber results in high wall stress during the entire cardiac cycle that leads to further distortions of ventricular size and shape causing a further deterioration in ventricular function [37].

In addition, animal studies clearly show that, once initiated, adverse ventricular remodeling continues even after histologic resolution to fibrous tissue in the infarcted region. Alterations of the genetic expression of myocytes in the remote noninfarcted area were consistent with the fetal pattern seen with cardiac hypertrophy produced by experimental hyperfunctional conditions [38]. Moreover, there was an increase in proportion of cells exhibiting apoptosis and an augmentation in interstitial fibrosis – both indicative that this initial regional loss of myocytes had triggered a more global detrimental response.

The discovery that this process of ventricular enlargement could be mitigated by long-term treatment with an angiotensin-converting enzyme (ACE) inhibitor provided a new therapeutic target beyond risk factor modification following myocardial infarction [39]. In animal studies, it was possible to demonstrate that long-term therapy with captopril reduced the progressive increase in ventricular chamber volume that occurred as a consequence of a myocardial infarction. Although infarct size was the greatest determinant of the extent of ventricular enlargement, treatment with the ACE inhibitor modified the time-dependent process of remodeling, resulting in a smaller ventricle chamber and less deterioration of pump performance [39]. In addition, in this animal model, the 1-year survival was also improved by ACE inhibitor therapy [40].

Changes in the size and shape of the ventricle as a consequence of a myocardial infarction are now clinically well recognized. As in animals, the loss of myocardium alters the regional and global wall stresses, which promote further distortions of the ventricular chamber [41]. Importantly, the extent of ventricular remodeling has been related to the risk of subsequent death and/or the development of heart failure [42, 43]. However, in the clinical arena, this remodeling response is highly variable. As anticipated, those with more extensive necrosis, greater interruption in coronary flow, and reduced myocardial perfusion are more likely to exhibit more adverse remodeling [44]. Unfortunately, these factors are not sufficiently predictive in the early infarct period to reliably identify on an individual basis patients that will have marked remodeling.

Indeed, almost one quarter of the patients experiencing a first ST-segment elevation anterior myocardial infarction treated early with contemporary therapies have almost normal ventricular function and size as assessed by echocardiography at 90 days [45]. This recovery in some but clearly not all patients with documented myocardial necrosis may reflect the marked variability of intrinsic cardiac regenerative capacity. The challenge for future investigative studies will be to identify those on the path for progressive remodeling and deterioration of function and to determine whether experimental procedures and therapies designed to augment cardiac regenerative capacity can reverse adverse ventricular remodeling and thereby improve prognosis.

Regenerative cardiovascular therapy is in its infancy and has many parallels with myocardial infarct size limitation. It has been over 30 years since experimental studies in animals first demonstrated that myocardial infarct size could be modified. From concept to clinical translation, providing definitive proof of the morbidity and mortality benefits of pharmacologic and invasive strategies targeting myocyte salvage required approximately 15 years. Indeed, this still remains a current area of intense investigation, with important fine-tuning studies still identifying optimal combinations of therapy to most promptly restore coronary patency and, more recently, to improve tissue perfusion. These past three decades of investigative work to limit myocardial damage should serve as a template for viewing the future of this exciting new therapeutic area of restoring functional myocardial tissue. We have crossed another paradigm changing threshold where animal studies have demonstrated cardiac regenerative capacity [46]. The journey to the clinical finish line of improving prognosis following myocardial infarction will also take decades of high-quality basic and clinical investigations to better understand the mechanisms of myocardial regeneration, and by this activity, optimize what will undoubtedly be multiple combinations of therapeutic approaches.

References

1 Swan HJC, Forrester JS, Diamond G, et al. Hemodynamic spectrum of myocardial infarction and cardiogenic shock. A conceptual model. Circulation 1972; 45:1097–1110.

2 Killip T, Kimball JT. Treatment of myocardial infarction in a coronary care unit. A two year experience with 250 patients. Am J Cardiol 1967; 20:457–464.

3 Geltman EM, Ehsani AA, Campbell MK, et al. The influence of location and extent of myocardial infarction on long-term ventricular dysrhythmia and mortality. Circulation 1979; 60:805–814.

4 Braunwald E. Thirteenth Bowditch lecture. The determinants of myocardial oxygen consumption. Physiologist 1969; 12:65–93.

5 Reimer KA, Jennings RB. The changing anatomic reference base of evolving myocardial infarction. Underestimation of myocardial collateral blood flow and overestimation of experimental anatomic infarct size due to tissue edema, hemorrhage and acute inflammation. Circulation 1979; 60:866–876.

6 Radvany P, Maroko PR, Braunwald E. Effects of hypoxemia on the extent of myocardial necrosis after experimental coronary occlusion. Am J Cardiol 1975; 35:795–800.

7 Maroko PR, Radvany P, Braunwald E, Hale SL. Reduction of infarct size by oxygen inhalation following acute coronary occlusion. Circulation 1975; 52:360–368.

8 Maroko PR, Libby P, Covell JW, et al. Precordial S-T segment elevation mapping: an atraumatic method for assessing alterations in the extent of myocardial ischemic injury. The effects of pharmacologic and hemodynamic interventions. Am J Cardiol 1972; 29:223–230.

9 Braunwald E, Maroko PR. The reduction of infarct size – an idea whose time (for testing) has come. Circulation 1974; 50:206–209.

10 Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI). Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. Lancet 1986; 1:397–402.

11 ISIS-2. Randomized trial of intravenous streptokinase, oral aspirin, both, or neither among 17 187 cases of suspected acute myocardial infarction: ISIS-2. Lancet 1988; 2:349–360.

12 Grines CL, O'Neill WW, Anselmo EG, et al. Comparison of left ventricular function and contractile reserve after successful recanalization by thrombolysis versus rescue percutaneous transluminal coronary angioplasty for acute myocardial infarction. Am J Cardiol 1988; 62:352–357.

13 O'Neill W, Timmis GC, Bourdillon PD, et al. A prospective randomized clinical trial of intracoronary streptokinase versus coronary angioplasty for acute myocardial infarction. N Engl J Med 1986; 314:812–818.

14 Antman EM, Anbe DT, Armstrong PW, et al. ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction – executive summary: a report of the American College of Cardiology/American

Heart Association Task Force on Practice Guidelines (writing committee to revise the 1999 guidelines for the management of patients with acute myocardial infarction). Circulation 2004; 110:588–636.

15 Sabatine MS, Cannon CP, Gibson CM, et al. Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med 2005; 352:1179–1189.

16 Chen ZM, Jiang LX, Chen YP, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet 2005; 366:1607–1621.

17 Antman EM, Morrow DA, McCabe CH, et al. Enoxaparin versus unfractionated heparin with fibrinolysis for ST-elevation myocardial infarction. N Engl J Med 2006; 354:1477–1488.

18 Yusuf S, Mehta SR, Chrolavicius S, et al. Comparison of fondaparinux and enoxaparin in acute coronary syndromes. N Engl J Med 2006; 354:1464–1476.

19 Anavekar NS, McMurray JJ, Velazquez EJ, et al. Relation between renal dysfunction and cardiovascular outcomes after myocardial infarction. N Engl J Med 2004; 351:1285–1295.

20 Kenchaiah S, Davis BR, Braunwald E, et al. Antecedent hypertension and the effect of captopril on the risk of adverse cardiovascular outcomes after acute myocardial infarction with left ventricular systolic dysfunction: insights from the survival and ventricular enlargement trial. Am Heart J 2004; 148:356–364.

21 Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. Acute Infarction Ramipril Efficacy (AIRE) Study Investigators. Lancet 1993; 342:821–828.

22 Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005; 366:1267–1278.

23 Anavekar NS, Gans DJ, Berl T, et al. Predictors of cardiovascular events in patients with type 2 diabetic nephropathy and hypertension: a case for albuminuria. Kidney Int Suppl, Vol 92:2004: 92:S50–S55.

24 Tonelli M, Isles C, Craven T, et al. Effect of pravastatin on rate of kidney function loss in people with or at risk for coronary disease. Circulation 2005; 112:171–178.

25 Solomon SD, Zelenkofske S, McMurray JJ, et al. Sudden death in patients with myocardial infarction and left ventricular dysfunction, heart failure, or both. N Engl J Med 2005; 352:2581–2588.

26 Smits PC, van Geuns RJ, Poldermans D, et al. Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. J Am Coll Cardiol 2003; 42:2063–2069.

27 Matsubara H. Risk to the coronary arteries of intracoronary stem cell infusion and G-CSF cytokine therapy. Lancet 2004; 363:746–747.

28 Gersh BJ, Simari RD. Cardiac cell-repair therapy: clinical issues. Nat Clin Pract Cardiovasc Med 2006; 3(suppl 1):S105–S109.

29 Kannel WB, Sorlie P, McNamara PM. Prognosis after initial myocardial infarction: the Framingham Study. Am J Cardiol 1979; 44:53–59.

30 Lewis EF, Moyé LA, Rouleau JL, et al. Predictors of late development of heart failure in stable survivors of myocardial infarction: the CARE study. J Am Coll Cardiol 2003; 42:1446–1453.

31 Pfeffer MA, Pfeffer JM, Fishbein MC, et al. Myocardial infarct size and ventricular function in rats. Circ Res 1979; 44:503–512.

32 Hutchins GM, Bulkley BH. Infarct expansion versus extension: two different complications of acute myocardial infarction. Am J Cardiol 1978; 41:1127–1132.

33 Fletcher PJ, Pfeffer JM, Pfeffer MA, Braunwald E. Left ventricular diastolic pressure–volume relations in rats with healed myocardial infarction. Effects on systolic function. Circ Res 1981; 49:618–626.

34 Pfeffer JM, Pfeffer MA, Fletcher PJ, Braunwald E. Ventricular performance in rats with myocardial infarction and failure. Am J Med 1984; 76:99–103.

35 Pfeffer JM, Pfeffer MA, Fletcher PJ, Braunwald E. Progressive ventricular remodeling in rat with myocardial infarction. Am J Physiol 1991; 260:H1406–H1414.

36 Solomon SD, Greaves SC, Rayan M, et al. Temporal dissociation of left ventricular function and remodeling following experimental myocardial infarction in rats. J Card Fail 1999; 5:213–223.

37 Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 1990; 81:1161–1172.

38 Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA. Controversies in ventricular remodelling. Lancet 2006; 367:356–367.

39 Pfeffer JM, Pfeffer MA, Braunwald E. Influence of chronic captopril therapy on the infarcted left ventricle of the rat. Circ Res 1985; 57:84–95.

40 Pfeffer JM, Pfeffer MA, Braunwald E. Hemodynamic benefits and prolonged survival with long-term captopril therapy in rats with myocardial infarction and heart failure. Circulation 1987; 75:I149–I155.

41 Mitchell GF, Lamas GA, Vaughan DE, Pfeffer MA. Left ventricular remodeling in the year after first anterior

myocardial infarction: a quantitative analysis of contractile segment lengths and ventricular shape. J Am Coll Cardiol 1992; 19:1136–1144.

42 White HD, Norris RM, Brown MA, et al. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. Circulation 1987; 76:44–51.

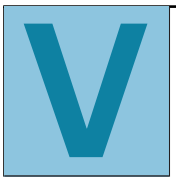
43 St John Sutton M, Pfeffer MA, Plappert T, et al. Quantitative two-dimensional echocardiographic measurements are major predictors of adverse cardiovascular events after acute myocardial infarction. The protective effects of captopril. Circulation 1994; 89:68–75.

44 Gaudron P, Eilles C, Kugler I, Ertl G. Progressive left ventricular dysfunction and remodeling after myocardial infarction: potential mechanisms and early predictors. Circulation 1993; 87:755–763.

45 Solomon SD, Glynn RJ, Greaves S, et al. Recovery of ventricular function after myocardial infarction in the reperfusion era: the healing and early afterload reducing therapy study. Ann Intern Med 2001; 134:451–458.

46 Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 2006; 113:1451–1463.

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman



PART V
Future directions

Cardiovascular Regeneration and Stem Cell Therapy
Edited by Annarosa Leri, Piero Anversa, William H. Frishman

22 CHAPTER 22

Conclusions – future directions

Piero Anversa, MD, *Edmund H. Sonnenblick,* MD, & *William H. Frishman,* MD

In April 2001, the recognition that hematopoietic stem cells (HSCs) can acquire the cardiac cell lineages and regenerate the infarcted heart in mice started a scientific revolution that has dominated and continues to dominate the cardiovascular field biologically and clinically [1]. Because of their potential ability to transdifferentiate into cardiomyocytes, vascular smooth muscle cells, and endothelial cells, HSCs have been proposed as a novel form of cell therapy for the damaged heart [2]. This possibility, however, has generated profound enthusiasm and strong skepticism in the scientific and clinical community. The basis for the controversy is multifactorial and involves not only scientific reasons but also emotional beliefs. Under the ingrained conviction that HSCs cannot disobey lineage specification, the detractors of HSC plasticity immediately claimed that they were unable to reproduce published results, and reaffirmed their negative view regarding the therapeutic efficacy of bone marrow cells for human disease [3]. In spite of the fact that the experimental design and methodological analysis of the myocardium used in these negative studies were strikingly different from those employed in the original report [1], these data fomented the debate and reproposed the use of skeletal myoblasts as the only safe and promising cells to be implemented in patients with chronic heart failure [4].

It is rather instructive that the disbelievers of HSC transdifferentiation are similarly against the possibility of cardiomyocyte regeneration by activation of endogenous stem cells [5]. Their conviction is that we are born with a determined number of cardiomyocytes that can live for 100 years or more. Since the majority of the cardiomyocytes are present at the death of the organ and organism, according to this paradigm, these cells should be essentially immortal. Because of this unshakable position, it has become convenient to interpret negative results as facts and positive data as the product of technical artifacts [3–5]. A good example can be found in a recent study in which the ability of multipotent adult progenitor cells (MAPCs) expressing green fluorescent protein (GFP) was studied [6]. These cells were labeled by bromodeoxyuridine (BrdU) prior to their injection in the brain on the assumption that BrdU-positive Purkinje neurons would be indicative of their origin from the implanted cells. However, the conclusion was reached that BrdU is a poor marker of transdifferentiation of primitive cells into neurons. Apparently, BrdU is lost from the dying injected GFP-positive MAPCs and incorporated by adjacent neurons falsely mimicking neuronal formation.

Although this report may simply reflect the poor viability of the administered cells, it was rapidly adopted [7] as the unquestionable demonstration of the misinterpretation of previous findings in favor of HSC plasticity [8, 9]. In fact, an identical approach was successfully employed to document the regeneration of neurons by spinal cord stem cells in which the issues of cell viability and labeling were carefully addressed [10]. In an identical manner, green fluorescence present in samples of skeletal muscle after immersion fixation with formalin was equated to autofluorescence of tissue sections stained with antibody against GFP [11]. This misunderstanding of autofluorescence was utilized to dismiss multiple evidence of HSC transdifferentiation in cardiomyocytes and coronary vessels [12].

Remarkably, the lack of GFP-positive Purkinje neurons in the study of the brain was assumed valid and the presence of BrdU labeled neurons an artifact [6]. However, the absence of GFP-positive cells strongly suggests that GFP staining was less than optimal. Properly implanted intact GFP-tagged cells should not disappear almost completely in 1 week, and, at minimum, a fraction of immature cells expressing GFP should have been found at the site of delivery in the treated brains. Alternatively, dead cells were injected and faded away quickly from the location of administration. Most importantly, the bone marrow cells previously shown to transdifferentiate into Purkinje neurons were freshly isolated from the bone marrow [8] or migrated directly from the bone marrow to the brain [9]. These bone marrow cells cannot be compared with MAPCs, which are a product of long-term culture [13]. Surprisingly, these alternative possibilities and dramatic differences were neither explored nor considered. So far, experimental studies and clinical trials have employed rather heterogeneous bone marrow cell preparations, but this treatment has resulted in a consistent improvement in function of the infarcted heart. However, the search for the most effective HSC for cardiac repair continues.

An unusual, unexpected behavior has emerged with the explosion of stem cell biology and the attempt to introduce cell therapy in the clinical management of heart failure. The scientific community is divided in several sectors, which tends to promote one cell type versus another. Embryonic stem cells (ESCs) are totipotent cells, which have a tremendous growth potential and can differentiate into cells of the ectoderm, endoderm, and mesoderm. ESCs are derived from the inner mass of the blastocyst and can be grown indefinitely *in vitro* [14]. Because of this characteristic, human ESC lines have been obtained from human blastocysts with the expectation of a future clinical application. Problems related to rejection and tumor formation exist with this cell population [15], but when these risks become resolved, extremely powerful cells will be available for the treatment of human diseases [16]. Currently, ESCs are a unique tool for the analysis of critical biological questions, while their implementation in patients remains to be seen in the future.

Mesenchymal stem cells (MSCs) have been defined as the nonhematopoietic progenitor cell compartment of the bone marrow. Marrow stromal cells are highly proliferative and clonogenic. MSCs possess a high degree of plasticity *in vitro* and *in vivo*. In the presence of growth factors, MSCs acquire multiple lineage cell phenotypes, including osteoblasts, adipocytes, chondrocytes, neuronal-like cells, endothelial cells, and cardiomyocytes [15]. The myocyte commitment of MSCs recapitulates, at least in part, embryonic development. Cultured MSCs become rod shaped and express progressively transcription factors and contractile proteins typical of cardiomyocytes. Cells beat spontaneously and synchronously as well as after stimulation with β-agonists [17]. Importantly, MSCs lead to myocardial regeneration *in vivo* [18, 19]. However, the intracoronary delivery of MSCs after infarction is coupled with fibroblast differentiation of the injected cells within the scar and myocyte formation within the surviving myocardium [15]. Additionally, engrafted MSCs may secrete peptides and have paracrine effects on the heart [20].

Circulating bone-marrow-derived cells include the subset of endothelial progenitor cells (EPCs), which promotes neovascularization *in vivo* [21]. EPCs have been derived from partly differentiated CD34-positive cells, immature CD133-positive HSCs, peripheral blood mononuclear cells, and CD14-positive monocytes. Although EPCs can be generated from different sources, they all show endothelial marker proteins such as vascular endothelial growth factor receptor 2 (KDR), von Willebrand factor, and endothelial nitric oxide synthase [22]. Delivery of bone-marrow-derived or circulating-blood-derived EPCs has a beneficial impact on postinfarction remodeling. Evidence suggests that EPCs generate new vessels and can induce migration and growth of c-kit-positive cardiac progenitor cells by secretion of specific cytokines [23]. Although experimentation *in vivo* remains to be performed, there is the possibility that EPCs, together with the local release of cytokines, activate the inherent regenerative capacity of the heart, reconstituting dead myocardium. Additionally, the ability of EPCs to acquire the myocyte lineage has been documented *in vitro* and *in vivo* [24, 25]. The clinical significance of these findings is enormous.

Several laboratories have identified regenerative resident progenitor cells in the adult heart [26, 27], but the controversy concerning the regeneration

potential of the heart is not resolved yet. However, these observations have led to the development of a new paradigm in which pluripotent cardiac stem cells (CSCs) are implicated in the turnover of cardiac cells and the formation of cardiomyocytes and coronary vessels following injury [28–31]. Unfortunately, CSCs cannot invade spontaneously the dead myocardium and repair the damaged heart. The recognition of factors enhancing the activation of the CSC pool [32], and their mobilization and translocation to pathological areas [33], where they eventually grow and differentiate, would make the previously impossible dream of therapeutic myocardial regeneration a feasible reality [34, 35].

In all cases, the reconstitution of the myocardium after infarction leads to a tissue that resembles the neonatal–fetal heart. Perhaps, the disruption of the extracellular matrix and/or the dramatic increase in diastolic or systolic stress opposes the restoration of the adult myocardial phenotype. This limitation may be overcome by the use of bioengineered scaffolds (see Chap. 18) loaded with peptides that enhance progenitor cell homing, proliferation, and differentiation into myocytes and coronary vasculature [36]. Genetic modifications of progenitor cells (see Chap. 19) can enhance their ability to survive within the hostile environment of the necrotic tissue, potentiate their growth, and ultimately favor their acquisition of committed progenies [37], leading to a rapid restoration of the injured myocardium. Stem cell therapy, gene therapy, and bioengineered scaffolds may be implemented together for the actual rebuilding of the failing heart. However, we do not know yet the optimal progenitor cell or the composition of the best mixture of progenitor cell classes that promote rapid and efficient cardiac repair. It is only by integrating the information discussed in the several sections of this book that fundamental issues of progenitor cell biology, function, and practical application will be resolved.

References

1 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 420:701–705.

2 Wollert KC, Drexler H. Clinical applications of stem cells for the heart. Circ Res 2005; 96:151–163.

3 Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol 2005; 23:845–856.

4 Chien KR. Stem cells: lost in translation. Nature 2004; 428:607–608.

5 Rubart M, Field LJ. Cardiac regeneration: repopulating the heart. Annu Rev Physiol 2006; 68:29–49.

6 Burns TC, Ortiz-González XR, Gutiérrez-Pérez M, et al. Thymidine analogs are transferred from pre-labeled donor to host cells in the central nervous system after transplantation: a word of caution. Stem Cells 2006; 24:1121–1127.

7 Pearson H. Stem cell tagging shows flaws. Nature 2006; 439:519.

8 Brazelton TR, Rossi FM, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 2000; 290:1775–1779.

9 Mezey E, Chandross KJ, Harta G, et al. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000; 290:1779–1883.

10 Shihabuddin LS, Horner PJ, Ray J, Gage FH. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. J Neurosci 2000; 20:8727–8735.

11 Jackson KA, Snyder DS, Goodell MA. Skeletal muscle fiber-specific green autofluorescence: potential for stem cell engraftment artifacts. Stem Cells 2004; 22:180–187.

12 International Society for Stem Cell Research. The Pulse: ISSCR Newsletter. Research Literature Highlights. Vol. 3:5 April 6, 2004.

13 Jiang Y, Jahagirdar BN, Lee Reinhardt R, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418:41–49.

14 He JQ, Ma Y, Lee Y, et al. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res 2003; 93:32–39.

15 Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005; 85:1373–1416.

16 Kofidis T, de Bruin JL, Yamane T, et al. Stimulation of paracrine pathways with growth factors enhances embryonic stem cell engraftment and host-specific differentiation in the heart after ischemic myocardial injury. Circulation 2005; 111:2486–2493.

17 Hakuno D, Fukuda K, Makino S, et al. Bone marrow–derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and muscarinic receptors. Circulation 2002; 105:380–386.

18 Kawada H, Fujita J, Kinjo K, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 2004; 104:3581–3587.

19 Nagawa N, Kangaya K, Itoh T, et al. Transplantation of mesenchymal stem cells improves cardiac function in a

rat model of dilated cardiomyopathy. Circulation 2005; 112:1128–1135.
20 Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005; 11:367–368.
21 Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 2004; 95:343–353.
22 Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275:964–966.
23 Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005; 39:733–742.
24 Koyanagi M, Urbich C, Chavakis E, et al. Differentiation of circulating endothelial progenitor cells to a cardiomyogenic phenotype depends on E-cadherin. FEBS Lett 2005; 579:6060–6066.
25 Murasawa S, Kawamoto A, Horii M, et al. Niche-dependent translineage commitment of endothelial progenitor cells, not cell fusion in general, into myocardial lineage cells. Arterioscler Thromb Vasc Biol 2005; 25: 1388–1394.
26 Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 2006; 113:1451–1463.
27 Bearzi C, Muller P, Amano K, et al. Identification and characterization of cardiac stem cells in the pig heart (abst). Circulation 2006; 114 (Suppl II): II-125.
28 Hosoda T, Bearzi C, Amano S, et al. Human cardiac progenitor cells regenerate cardiomyocytes and coronary vessels repairing the infarcted myocardium (abst). Circulation 2006; 114 (Suppl II): II-51.
29 Hosoda T, Amano S, Bearzi C, et al. The reconstitution of large coronary arteries by cardiac stem cells is mediated by hypoxia (abst). Circulation 2006; 114 (Suppl II): II-231.
30 Urbanek K, Cesselli D, Rota m, et al. Cardiac stem cell niches control cardiomyogenesis in the adult mouse heart (abst). Circulation 2006; 114 (Suppl II): II-238.
31 Bolli R, Jneid H, Tang X-L, et al. Intracoronary administration of cardiac stem cells improves cardiac function in pigs with old infarction (abst). Circulation 2006; 114 (Suppl II): II-239.
32 Tillmanns J, Rota M, Hosoda T, et al. IGF-1 and HGF promote the engratment of cardiac stem cells, which in turn regenerate the entire coronary vasculature and the dead myocardium after infarctions in rats (abst). Circulation 2006; 114 (Suppl II): II-164.
33 Boni A, Rota M, Siggins RW, et al. Cardiac progenitor cell migration after myocardial infarction (abst). Circulation 2006; 114 (Suppl II): II-163.
34 Rota M, De Angelis A, Tillmanns J, et al. Cardiac stem cells regenerate the coronary vasculature and the scarred infarcted myocardium in rats (abst). Circulation 2006; 114 (Suppl II): II-265.
35 Johnston P, Sasano T, Mills K, et al. Isolation, expansion and delivery or cardiac derived stem cells in a porcine model of myocardial infarction (abst). Circulation 2006; 114 (Suppl II): II-125.
36 Hsieh PCH, Davis ME, Lisowski LK, Lee RT. Endothelial-cardiomyocyte interactions in cardiac development and repair. Ann Rev Physiol 2006; 68:51–66.
37 Heleniak H, Baker M. Barlucchi L, et al. The nuclear localization of protein kinase B increases the number of myocytes in the heart of transgenic mice. Circulation 2003; 108:IV–287.

Index